

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|--|--|
| (51) International Patent Classification⁶ : C12N 15/63, 5/10, 15/09, 15/79, 5/00, A61K 48/00, 38/00 | A1 | (11) International Publication Number: WO 99/54485 (43) International Publication Date: 28 October 1999 (28.10.99) |
| (21) International Application Number: PCT/US99/08427 (22) International Filing Date: 16 April 1999 (16.04.99) (30) Priority Data: 09/062,365 17 April 1998 (17.04.98) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 09/062,365 (CON) Filed on 17 April 1998 (17.04.98) (71) Applicant (for all designated States except US): THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK [US/US]; West 116th Street and Broadway, New York, NY 10027 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SCHMIDT, Ann, Marie [US/US]; 242 Haven Road, Franklin Lakes, NJ 07417 (US). STERN, David [US/US]; 63 Tanners Road, Great Neck, NY 11020 (US). (74) Agent: WHITE, John, P.; Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, NY 10036 (US). | (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> | |
| (54) Title: A METHOD FOR INHIBITING TUMOR INVASION OR SPREADING IN A SUBJECT | | |
| (57) Abstract <p>The present invention provides for a method for inhibiting tumor invasion or metastasis in a subject which comprises administering to the subject a therapeutically effective amount of a form of soluble Receptor for Advanced Glycation Endproducts (RAGE). The present invention also provides a method for evaluating the ability of an agent to inhibit tumor invasion in a local cellular environment which comprises: (a) admixing with cell culture media an effective amount of the agent; (b) contacting a tumor cell in cell culture with the media from step (a); (c) determining the amount of spreading of the tumor cell culture, and (d) comparing the amount of spreading of the tumor cell culture determined in step (c) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit tumor invasion in the local cellular environment. The present invention also provides a pharmaceutical composition which comprises a therapeutically effective amount of the agent evaluated in the aforementioned method and a pharmaceutically acceptable carrier.</p> | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|----|--------------------------|----|--|----|--|----|--------------------------|
| AL | Albania | ES | Spain | LS | Lesotho | SI | Slovenia |
| AM | Armenia | FI | Finland | LT | Lithuania | SK | Slovakia |
| AT | Austria | FR | France | LU | Luxembourg | SN | Senegal |
| AU | Australia | GA | Gabon | LV | Latvia | SZ | Swaziland |
| AZ | Azerbaijan | GB | United Kingdom | MC | Monaco | TD | Chad |
| BA | Bosnia and Herzegovina | GE | Georgia | MD | Republic of Moldova | TG | Togo |
| BB | Barbados | GH | Ghana | MG | Madagascar | TJ | Tajikistan |
| BE | Belgium | GN | Guinea | MK | The former Yugoslav Republic of Macedonia | TM | Turkmenistan |
| BF | Burkina Faso | GR | Greece | ML | Mali | TR | Turkey |
| BG | Bulgaria | HU | Hungary | MN | Mongolia | TT | Trinidad and Tobago |
| BJ | Benin | IE | Ireland | MR | Mauritania | UA | Ukraine |
| BR | Brazil | IL | Israel | MW | Malawi | UG | Uganda |
| BY | Belarus | IS | Iceland | MX | Mexico | US | United States of America |
| CA | Canada | IT | Italy | NE | Niger | UZ | Uzbekistan |
| CF | Central African Republic | JP | Japan | NL | Netherlands | VN | Viet Nam |
| CG | Congo | KE | Kenya | NO | Norway | YU | Yugoslavia |
| CH | Switzerland | KG | Kyrgyzstan | NZ | New Zealand | ZW | Zimbabwe |
| CI | Côte d'Ivoire | KP | Democratic People's Republic of Korea | PL | Poland | | |
| CM | Cameroon | KR | Republic of Korea | PT | Portugal | | |
| CN | China | KZ | Kazakhstan | RO | Romania | | |
| CU | Cuba | LC | Saint Lucia | RU | Russian Federation | | |
| CZ | Czech Republic | LJ | Liechtenstein | SD | Sudan | | |
| DE | Germany | LK | Sri Lanka | SE | Sweden | | |
| DK | Denmark | LR | Liberia | SG | Singapore | | |
| EE | Estonia | | | | | | |

A Method For Inhibiting Tumor Invasion or Spreading in a
Subject

5 This application is a continuation-in-part application of
U.S. Serial No. 09/062,365, filed April 17, 1998, the
content of which is hereby incorporated by reference.

10 The invention disclosed herein was made with Government
support under National Institutes of Health Grant No.
AG00602 HL60901, HL56881 and DK52496 from the Department of
Health and Human Services and United States Public Health
Service. Accordingly, the U.S. Government has certain
rights in this invention.

15

Background of the Invention

Throughout this application, various publications are
referenced by author and date. Full citations for these
20 publications may be found listed alphabetically at the end
of the specification immediately preceding Sequence Listing
and the claims. The disclosures of these publications in
their entireties are hereby incorporated by reference into
this application in order to more fully describe the state
25 of the art as known to those skilled therein as of the date
of the invention described and claimed herein.

An important means by which tumors grow and invade
surrounding normal tissue is by a complex series of cell-
30 cell and cell matrix interactions. We have focused on the
interaction of tumor cells with matrix-associated
components. The Receptor to AGE (RAGE) interacts with a
range of physiologically and pathophysiologically-relevant
ligands (1-5). In normal developing neurons of the central
35 nervous system, the expression of RAGE is markedly enhanced
and co-localizes with that of its ligand, amphoterin.
Amphoterin, a matrix-associated polypeptide, is expressed in
developing neurons and certain tumor cells, such as rat C6
glioma cells (6-12).

Summary of the Invention

The present invention provides for a method for inhibiting tumor invasion or metastasis in a subject which comprises administering to the subject a therapeutically effective amount of a form of soluble Receptor for Advanced Glycation Endproducts (RAGE). The present invention also provides a method for evaluating the ability of an agent to inhibit tumor invasion in a local cellular environment which comprises: (a) admixing with cell culture media an effective amount of the agent; (b) contacting a tumor cell in cell culture with the media from step (a); (c) determining the amount of spreading of the tumor cell culture, and (d) comparing the amount of spreading of the tumor cell culture determined in step (c) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit tumor invasion in the local cellular environment. The present invention also provides a pharmaceutical composition which comprises a therapeutically effective amount of the agent evaluated in the aforementioned method and a pharmaceutically acceptable carrier.

Brief Description of the Figures

Figure 1. Transfection of C6 glioma cells with constructs expressing sRAGE results in diminished tumor volume compared with mock-transfected tumor cells. 1 X 10⁵ tumor cells/ml (0.1 ml) were injected into the backs of nude mice. Cells were previously transfected either with constructs overexpressing sRAGE or mock-transfection (vector alone) as described above. At the indicated days after tumor injection, tumor volume was measured. The mean \pm standard error of the mean is reported. Twenty mice were studied per experimental group. Statistical considerations are indicated. This experiment was repeated three times with analogous results.

Figure 2. The growth rate of sRAGE-transfected tumor cells is not different than that of mock-transfected tumor cells, *in vivo*. The rate of tumor cell growth was calculated and the slope of the line determined (\pm standard error) using Microsoft Excel. The y axis represents the natural logarithm of the tumor volume and the x axis represents time (days). These studies indicated that the slope of the growth curve for mock-transfected tumor cells was 0.247 ± 0.025 . For sRAGE-transfected tumor cells, the slope of the growth curve was 0.241 ± 0.029 .

Figure 3. The rate of growth of sRAGE-transfected tumor cells is similar to that of mock-transfected tumor cells in *in vitro* studies. C6 glioma cells transfected with either sRAGE or mock were placed into 96 well tissue culture wells. On day three after onset of incubation, cells were fixed and stained and relative cell number assessed using the CYQUANT[®] assay from AMERSHAM[®]. These studies indicated that the relative cell number of sRAGE-transfected C6 cells on day three was 0.90 ± 0.03 and 1.0 ± 0.04 in mock-transfected C6 tumor cells ($p=0.11$).

-4-

Figure 4. Systemic administration of sRAGE results in diminished tumor volume in C6 glioma cells injected into nude mice. C6 glioma cells ($1 \times 10^5/\text{ml}$; 0.1 ml) were injected into the backs of nude mice. Immediately after injection, once daily injections of either mouse sRAGE (20 or 2 $\mu\text{g}/\text{day}$) or mouse serum albumin (MSA; 40 $\mu\text{g}/\text{day}$) were begun intraperitoneally. Tumor volume was measured on days 14 and 17 after injection. For each experimental group, n=10. Statistical considerations are indicated.

10

Figure 5. The rate of growth of C6 glioma cells is similar in sRAGE and MSA-treated mice *in vivo*. The rate of tumor cell growth was calculated and the slope of the line determined (\pm standard error) using Microsoft Excel. The y axis represents the natural logarithm of the tumor volume and the X axis represents time (days). The slope of the growth curve for C6 glioma tumor cells grown in the presence of mouse serum albumin was 0.264 ± 0.03 . The slopes of the growth curve for C6 glioma cells grown in the presence of sRAGE (20 vs. 2 $\mu\text{g}/\text{ml}$) were not different (0.311 ± 0.04 and 0.313 ± 0.04 , respectively).

15

Figure 6. The rate of growth of tumor cells is similar in the presence of either sRAGE or mouse serum albumin in *in vitro* studies. C6 glioma cells were placed into 96 well tissue culture wells and grown in the presence of either sRAGE (20 or 2 $\mu\text{g}/\text{ml}$) or mouse serum albumin (40 $\mu\text{g}/\text{ml}$). On day three after onset of incubation, cells were fixed and stained and relative cell number assessed using the CYQUANT® assay from AMERSHAM®. Relative cell number (day three) of cells treated with MSA was 1.0 ± 0.045 . Similarly, relative cell number in the presence of sRAGE (20 or 2 $\mu\text{g}/\text{ml}$) was 1.03 ± 0.06 and 1.02 ± 0.06 , respectively. Statistical considerations are shown.

25

Figure 7. RAGE and amphotericin are expressed in tumor cells (A); effect of stably-transfected RAGE/RAGE mutant rat C6 glioma cells (B-F); soluble RAGE (G); or

30

35

-5-

anti-RAGE/amphoterin F(ab')₂ (H) in a model of local tumor growth and invasion. (A1-2). RAGE (1) and amphoterin (2) are expressed in tumor cells. Extracts of murine Lewis lung carcinoma cells (lane 2) and rat C6 glioma cells (lane 3) were prepared and subjected to SDS-PAGE and immunoblotting with anti-RAGE IgG (A1) or anti-amphoterin IgG (A2) as described. In A1, murine soluble recombinant RAGE (0.5 µg) was employed as positive control (lane 1); in A2, recombinant rat amphoterin (0.5 µg) was employed (lane 1). Molecular weight markers run simultaneously are indicated in the left margin (kDa). Controls employing preimmune rabbit IgG were negative (not shown). (B-F). Effect of stably-transfected RAGE/RAGE mutant C6 glioma cells implanted into immunocompromised (nude) mice. (B). The indicated clones of RAGE/RAGE mutant C6 glioma cells were injected in the dorsal midline of nude mice. Tumors were followed serially and mice sacrificed on day 21. Mean tumor volume (mm³) ± standard error (SE) on day 21 is shown. Statistical considerations are as follows compared with mean tumor volume of three mock-transfected clones (pooled) (n=17): full-length RAGE (pooled) (n=18) vs mock, p<0.001; tail-deletion RAGE (pooled) (n=21) vs mock, p<0.01; and soluble RAGE (pooled) (n=18) vs mock, p<0.001. (C-F). Representative photographs of NCR nude mice-bearing mock (C) or RAGE/RAGE mutant C6 glioma clones on day 21 after implantation: (D) full-length RAGE, F2; (E) tail-deletion RAGE, T2; and (F), soluble RAGE, S1. F2, T2 and S1 refer to the particular clones as identified in figure 1A. (G). Effect of administration of soluble RAGE. Immediately upon injection of wild-type rat C6 glioma cells into the dorsal midline of NCR nude mice, the indicated doses of either murine sRAGE or murine serum albumin (MSA) were begun and continued daily until sacrifice, day 21. Mean tumor volume (mm³) was measured on days 14, 17 and 21 after implantation; mean tumor volume ± SE is shown, n=10 per group. Statistical considerations are indicated in the figure. (H). Effect of anti-RAGE/amphoterin F(ab')₂. Mice with severe combined immunodeficiency were injected with

-6-

wild-type rat C6 glioma cells into the dorsal midline. Immediately upon implantation of tumor cells, the indicated F(ab')₂ fragments of rabbit nonimmune, anti-RAGE, and/or anti-amphoterin IgG were administered every 3 days by intraperitoneal injection until day 15. Mean tumor volume (mm³) ± SE on day 21 is shown, n= 7 per group. Statistical considerations are indicated in the figure.

Figure 8. Blockade of RAGE suppresses distant metastasis. (A). Effect of administration of sRAGE on numbers of lung surface metastases. Murine Lewis lung carcinoma cells were injected into the dorsal midline of C57BL/6J mice. On day 14, removal of primary tumor was performed. Three days prior to removal of primary tumor, mice received the indicated dose of sRAGE or MSA once daily by intraperitoneal injection until sacrifice, 21 days after removal of primary tumor. At that time, numbers of lung surface metastases were counted. Mean number of lung surface metastases ± SE is shown, n=9 per group. (B-E). Representative photographs of lungs removed from C57BL/6J mice bearing metastatic Lewis lung carcinoma. (B) MSA, 200 µg IP/day; (C) sRAGE, 2 µg IP/day; (D) sRAGE 20 µg IP/day; and (E) sRAGE, 100 µg IP/day. (F-G). Representative sections from lung parenchyma were retrieved from C57BL/6J mice bearing metastatic Lewis lung carcinoma. Sections (5 µm thick) were fixed in formalin, embedded in paraffin and then subjected to staining with hematoxylin and eosin. (F) MSA, 200 µg IP/day; and (G) murine sRAGE, 100 µg IP/day. Magnification: x2.

Figure 9. Amphoterin/RAGE mediate tumor cell migration and invasion: in vitro analyses. (A). Effect of sRAGE on tumor cell proliferation *in vitro*. Wild-type rat C6 glioma cells were cultured in the presence of the indicated concentrations of MSA or murine sRAGE. Three days after plating, cell number was assessed using the CyQUANT cell proliferation assay as described. Mean number of cells ± SE in 16 wells is reported. Statistical considerations are

-7-

indicated. (B). Effect of RAGE/RAGE mutation in clones of stably-transfected rat C6 glioma cells on tumor cell invasion. Invasion assays were performed as described employing three different clones of stably-transfected RAGE/RAGE mutant C6 glioma cells. The number of cells invading matrigel \pm SE in 10 high-powered fields (hpf) is reported. Statistical considerations are as follows compared with number of cells invading Matrigel from three mock-transfected clones (pooled): full-length RAGE (pooled) vs mock, $p < 0.00001$; tail-deletion RAGE (pooled) vs mock, $p < 0.00001$; and soluble RAGE (pooled) vs mock, $p < 0.00001$. (C-D). Effect of RAGE/amphotericin blockade on tumor cell invasion. Invasion assays employing rat C6 glioma cells were performed in the presence of the indicated concentrations of MSA or sRAGE (C) or anti-RAGE/anti-amphotericin IgG (D). The number of cells invading matrigel \pm SE in 10 hpf is reported. Statistical considerations are indicated. (E). Effect of RAGE/RAGE mutation in clones of stably-transfected rat C6 glioma cells on tumor cell migration. Migration assays were performed as described employing three different clones of stably-transfected RAGE/RAGE mutant C6 glioma cells. The number of migrating cells \pm standard error in 10 hpf is reported. Statistical considerations are as follows compared with number of migrating cells from three mock-transfected clones (pooled): full-length RAGE (pooled) vs mock, $p < 0.00001$; tail-deletion RAGE (pooled) vs mock, $p < 0.00001$; and soluble RAGE (pooled) vs mock, $p < 0.00001$. (F-G). Effect of RAGE/amphotericin blockade on tumor cell migration. Migration assays employing rat C6 glioma cells were performed in the presence or absence of the indicated concentrations of MSA or sRAGE (F) or anti-RAGE/anti-amphotericin IgG (G). The number of invading cells \pm SE in 10 hpf is reported. Statistical considerations are indicated.

35

-8-

Figure 10. Lack of effect of RAGE blockade on tumor attachment (A), angiogenesis (B), collagenase (C) or plasmin (D) activity. (A). Attachment assay. Rat C6 glioma cells were employed in an in vitro assay of tumor attachment as described in the presence of sRAGE or MSA. At the indicated times, wells were depleted of medium by aspiration and washed twice. At each point, cell number per well was quantified using the CyQUANT assay kit. The mean % of cells attached compared with total number added \pm SE of 16 wells is shown. No statistically-significant difference was observed between cells in the presence of MSA vs sRAGE ($p>0.1$). (B). Angiogenesis. Hydron pellets coated with basic FGF as described were implanted into the corneal pockets of C57BL/6J mice. Immediately after implantation, mice were treated once daily intraperitoneally with the indicated dose of MSA or sRAGE until sacrifice on day 5, $n=8$ per group. The eyes were then examined under slit-lamp biomicroscopy and area of angiogenic response calculated. Mean angiogenic area \pm SE is reported. Statistical considerations are indicated. (C). Assessment of collagenase activity. Activity of type IV collagenase was determined employing rat C6 glioma cells as described in the presence of the indicated concentrations of either MSA or sRAGE. Mean activity \pm SE is shown in 8 wells per group. At each time point, no statistically-significant difference was observed between cells in the presence of MSA vs sRAGE ($p>0.2$). (D). Assessment of plasmin activity. Activity of plasmin was determined employing rat C6 glioma cells as described in the presence of the indicated concentrations of either MSA or sRAGE. Mean plasmin activity \pm SE is shown in 3 wells per group. At each time point, no statistically-significant difference was observed between cells in the presence of MSA vs sRAGE ($p>0.1$).

Detailed Description of the Invention

The present invention provides for a method for inhibiting tumor invasion or metastasis in a subject which comprises administering to the subject a therapeutically effective amount of a form of soluble Receptor for Advanced Glycation Endproducts (RAGE).

As used herein, a "form of soluble RAGE" encompasses peptides which are derived from naturally occurring RAGE protein. The following are examples of forms of soluble RAGE: mature human soluble RAGE, mature bovine soluble RAGE, mature murine soluble RAGE, fragments or portions of a soluble RAGE. Representative peptides of the present invention include but are not limited to peptides having an amino acid sequence which corresponds to amino acid numbers (2-30), (5-35), (10-40), (15-45), (20-50), (25-55), (30-60), (30-65), (10-60), (8-100), (14-75), (24-80), (33-75), (45-110) of human sRAGE protein. In one embodiment the form of soluble RAGE may consist essentially of any such portion of the human RAGE peptide. In another embodiment, the form of soluble RAGE may consist essentially of a peptide which comprises any such portion of the human RAGE amino acid having the sequence from amino acid at position 1 (alanine) to amino acid number 332 (alanine) (without considering the 22 amino acid leader sequence).

The abbreviations used herein for amino acids are those abbreviations which are conventionally used: A = Ala = Alanine; R = Arg = Arginine; N = Asn = Asparagine; D = Asp = Aspartic acid; C = Cys = Cysteine; Q = Gln = Glutamine; E = Glu = Glutamic acid; G = Gly = Glycine; H = His = Histidine; I = Ile = Isoleucine; L = Leu = Leucine; K = Lys = Lysine; M = Met = Methionine; F = Phe = Phenylalanine; P = Pro = Proline; S = Ser = Serine; T = Thr = Threonine; W = Trp = Tryptophan; Y = Tyr = Tyrosine; V = Val = Valine. The amino acids may be L- or D- amino acids. An amino acid may be replaced by a synthetic amino acid which is altered so as

-10-

to increase the half-life of the peptide or to increase the potency of the peptide, or to increase the bioavailability of the peptide.

5 In one embodiment the form of soluble RAGE comprises a peptide having the sequence from alanine at position 1 to alanine at position 332 of human RAGE.

10 In another embodiment the form of soluble RAGE comprises a peptide of 10-40 amino acids having a sequence taken from within the sequence from alanine at position 1 to alanine at position 332 of human RAGE.

15 In another embodiment, the form of soluble RAGE comprises a peptide having the following sequence of mature human RAGE.

The following is the amino acid sequence of mature human RAGE (which does not include a 22 amino acid leader sequence):

20 Ala Gln Asn Ile Thr Ala Arg Ile Gly Glu Pro Leu Val Leu Lys
Cys Lys Gly Ala Pro Lys Lys Pro Pro Gln Arg Leu Glu Trp Lys
Leu Asn Thr Gly Arg Thr Glu Ala Trp Lys Val Leu Ser Pro Gln
Gly Gly Gly Pro Trp Asp Ser Val Ala Arg Val Leu Pro Asn Gly
Ser Leu Phe Leu Pro Ala Val Gly Ile Gln Asp Glu Gly Ile Phe
25 Arg Cys Gln Ala Met Asn Arg Asn Gly Lys Glu Thr Lys Ser Asn
Tyr Arg Val Arg Val Tyr Gln Ile Pro Gly Lys Pro Glu Ile Val
Asp Ser Ala Ser Glu Leu Thr Ala Gly Val Pro Asn Lys Val Gly
Thr Cys Val Ser Glu Gly Ser Tyr Pro Ala Gly Thr Leu Ser Trp
His Leu Asp Gly Lys Pro Leu Val Pro Asn Glu Lys Gly Val Ser
30 Val Lys Glu Gln Thr Arg Arg His Pro Glu Thr Gly Leu Phe Thr
Leu Gln Ser Glu Leu Met Val Thr Pro Ala Arg Gly Gly Asp Pro
Arg Pro Thr Phe Ser Cys Ser Phe Ser Pro Gly Leu Pro Arg His
Arg Ala Leu Arg Thr Ala Pro Ile Gln Pro Arg Val Trp Glu Pro
Val Pro Leu Glu Glu Val Gln Leu Val Val Glu Pro Glu Gly Gly
35 Ala Val Ala Pro Gly Gly Thr Val Thr Leu Thr Cys Glu Val Pro
Ala Gln Pro Ser Pro Gln Ile His Trp Met Lys Asp Gly Val Pro
Leu Pro Leu Pro Pro Ser Pro Val Leu Ile Leu Pro Glu Ile Gly
Pro Gln Asp Gln Gly Thr Tyr Ser Cys Val Ala Thr His Ser Ser

-11-

His Gly Pro Gln Glu Ser Arg Ala Val Ser Ile Ser Ile Ile Glu
Pro Gly Glu Glu Gly Pro Thr Ala Gly Ser Val Gly Gly Ser Gly
Leu Gly Thr Leu Ala Leu Ala Leu Gly Ile Leu Gly Gly Leu Gly
Thr Ala (Seq I.D. No. 1).

5

In one embodiment the form of soluble RAGE comprises a V
domain of naturally occurring soluble RAGE. In another
embodiment the form of soluble RAGE comprises a C domain of
naturally occurring soluble RAGE. The 22 amino acid leader
10 sequence of immature human RAGE is Met Ala Ala Gly Thr Ala
Val Gly Ala Trp Val Leu Val Leu Ser Leu Trp Gly Ala Val Val
Gly (Seq. I.D. No. 2).

In one embodiment the form of soluble RAGE is a peptide
15 expressed by a replicable vector containing nucleic acid
encoding the form of soluble RAGE. In one embodiment the
form of soluble RAGE is a peptide which corresponds to all
or part of soluble RAGE expressed by a replicable vector
containing nucleic acid encoding the form of soluble RAGE.
20 In another embodiment the replicable vector is capable of
expressing the peptide within a tumor cell in a subject.

In another embodiment the tumor cell is a eukaryotic cell.

25 In another embodiment the replicable vector is a plasmid, an
attenuated virus, a phage, a phagemid or a linear nucleic
acid.

In another embodiment, the method further comprises
30 administering a pharmaceutically acceptable carrier to the
subject during the administration of the form of soluble
RAGE.

In one embodiment the administration is via intralesional,
35 intraperitoneal, intramuscular or intravenous injection;
infusion; intrathecal administration; subcutaneous
administration; liposome-mediated delivery; or topical,
nasal, oral, ocular or otic delivery. As used herein,

-12-

intrathecal administration includes administration in the cerebrospinal fluid by lumbar puncture.

5 In one embodiment of the present invention, the subject is a mammal. In another embodiment, the mammal is a human.

10 In one embodiment, the form of soluble RAGE is administered daily, weekly or monthly. The form of soluble RAGE may be delivered hourly, daily, weekly, monthly, yearly (e.g. in a time release form) or as a one time delivery. The delivery may be continuous delivery for a period of time, e.g. intravenous delivery. The agent or pharmaceutical composition of the present invention may be delivered intercranially or into the spinal fluid. In another
15 embodiment, the therapeutically effective amount comprises a dose from about 0.000001 mg/kg body weight to about 100 mg/kg body weight. In a preferred embodiment, the therapeutically effective amount comprises a dose of from about 100 ng/day/kg body weight to about 200 mg/day/kg body weight.
20 weight.

The present invention also provides a method for evaluating the ability of an agent to inhibit tumor invasion in a local cellular environment which comprises: (a) admixing with cell
25 culture media an effective amount of the agent; (b) contacting a tumor cell in cell culture with the media from step (a); (c) determining the amount of spreading of the tumor cell culture, and (d) comparing the amount of spreading of the tumor cell culture determined in step (c)
30 with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit tumor invasion in the local cellular environment.

35 In one embodiment the tumor cell is a eukaryotic cell. In another embodiment the tumor cell is a cell of a subject. In a further embodiment, the subject is a human, a mouse, a rat, a dog or a non-human primate.

-13-

In a further embodiment, the agent comprises a peptide, a peptidomimetic, a nucleic acid, a synthetic organic molecule, an inorganic molecule, a carbohydrate, a lipid, an antibody or fragment thereof, or a small molecule. The antibody may be a monoclonal antibody. The antibody may be a polyclonal antibody. In one embodiment, the fragment of the antibody comprises a Fab fragment. In another embodiment the fragment of the antibody comprises a complementarity determining region or a variable region. The agent may be conjugated to a carrier. The peptide or agent may be linked to an antibody, such as a Fab or a Fc fragment for specifically targeted delivery.

In one embodiment the peptide is a synthetic peptide or a peptide analog. The peptide may be a non-natural peptide which has chirality not found in nature, i.e. D- amino acids or L-amino acids. In another embodiment the peptide comprises at least a portion of the sequence -Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val- (Seq. I.D. No. 3).

In a further embodiment the peptide comprises at least a portion of the sequence -Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met- (Seq. I.D. No. 4). In another embodiment the peptide has the amino acid sequence A-Q-N-I-T-A-R-I-G-E-P-L-V-L-K-C-K-G-A-P-K-K-P-P-Q-R-L-E-W-K (Seq. I.D. No. 5).

In another embodiment the peptide has the amino acid sequence A-Q-N-I-T-A-R-I-G-E (Seq. I.D. No. 6).

In addition to naturally-occurring forms of soluble RAGE, the present invention also embraces other peptides such as peptide analogs of sRAGE. Such analogs include fragments of sRAGE. Following the procedures of the published application by Alton et al. (WO 83/04053), one can readily design and manufacture genes coding for microbial expression of peptides having primary conformations which differ from

-14-

that herein specified for in terms of the identity or location of one or more residues (e.g., substitutions, terminal and intermediate additions and deletions). Alternately, modifications of cDNA and genomic genes can be readily accomplished by well-known site-directed mutagenesis techniques and employed to generate analogs and derivatives of sRAGE polypeptide. Such products share at least one of the biological properties of sRAGE but may differ in others. As examples, products of the invention include those which are foreshortened by e.g., deletions; or those which are more stable to hydrolysis (and, therefore, may have more pronounced or longerlasting effects than naturally-occurring); or which have been altered to delete or to add one or more potential sites for O-glycosylation and/or N-glycosylation or which have one or more cysteine residues deleted or replaced by e.g., alanine or serine residues and are potentially more easily isolated in active form from microbial systems; or which have one or more tyrosine residues replaced by phenylalanine and bind more or less readily to target proteins or to receptors on target cells. Also comprehended are polypeptide fragments duplicating only a part of the continuous amino acid sequence or secondary conformations within sRAGE, which fragments may possess one property of sRAGE and not others. It is noteworthy that activity is not necessary for any one or more of the polypeptides of the invention to have therapeutic utility or utility in other contexts, such as in assays of sRAGE antagonism. Competitive antagonists may be quite useful in, for example, cases of overproduction of sRAGE.

30

The agent of the present invention may be a peptidomimetic which may be at least partially unnatural. The agent may be a small molecule mimic of a portion of the amino acid sequence of sRAGE. The agent may have increased stability, efficacy, potency and bioavailability by virtue of the mimic. Further, the agent may have decreased toxicity. The peptidomimetic agent may have enhanced mucosal intestinal permeability. The agent may be synthetically prepared. The

35

-15-

agent of the present invention may include L-, D-, DL- or unnatural amino acids, alpha, alpha-disubstituted amino acids, N-alkyl amino acids, lactic acid (an isoelectronic analog of alanine). The peptide backbone of the agent may have at least one bond replaced with PSI-[CH=CH] (Kempf et al. 1991). The agent may further include trifluorotyrosine, p-Cl-phenylalanine, p-Br-phenylalanine, poly-L-propargylglycine, poly-D,L-allyl glycine, or poly-L-allyl glycine. Examples of unnatural amino acids which may be suitable amino acid mimics include β -alanine, L- α -amino butyric acid, L- γ -amino butyric acid, L- α -amino isobutyric acid, L- ϵ -amino caproic acid, 7-amino heptanoic acid, L-aspartic acid, L-glutamic acid, cysteine (acetamindomethyl), N- ϵ -Boc-N- α -CBZ-L-lysine, N- ϵ -Boc-N- α -Fmoc-L-lysine, L-methionine sulfone, L-norleucine, L-norvaline, N- α -Boc-N- δ CBZ-L-ornithine, N- δ -Boc-N- α -CBZ-L-ornithine, Boc-p-nitro-L-phenylalanine, Boc-hydroxyproline, Boc-L-thiopropine. (Blondelle, et al. 1994; Pinilla, et al. 1995).

In one embodiment of the present invention the agent is a form of soluble human RAGE. In another embodiment the agent is an extracellular portion human RAGE. In another embodiment the agent inhibits an interaction between the tumor cell and an extracellular matrix molecule. In one embodiment the extracellular matrix molecule is a laminin, a fibronectin, amphoterin, a cadherin, an integrin or a hyaluronic acid. In another embodiment the integrin is an $\alpha V \beta V$ integrin, an $\alpha V \beta III$ integrin, or an $\alpha I \beta II$ integrin. In another embodiment, the laminin is $\beta 1$ laminin. In another embodiment the agent inhibits binding of RAGE to amphoterin.

The present invention also provides a pharmaceutical composition which comprises a therapeutically effective amount of the agent evaluated in the aforementioned method and a pharmaceutically acceptable carrier. In one embodiment the carrier is a diluent, an aerosol, a topical carrier, an aqueous solution, a replicable nucleic acid vector, a

-16-

liposome, a magnetic bead, a nonaqueous solution or a solid carrier.

5 The actual effective amount will be based upon the size of the subject. The biodegradability of the agent, the bioactivity of the agent and the bioavailability of the agent are factors which will alter the effective amount. The agent may be delivered topically in a creme or salve carrier. It may be reapplied as needed based upon the
10 absorbancy of the carrier to the skin or mucosa or wound. If the agent does not degrade quickly, is bioavailable and highly active, a smaller amount will be required to be effective. The effective amount will be known to one of skill in the art; it will also be dependent upon the form of
15 the agent, the size of the agent and the bioactivity of the agent. One of skill in the art could routinely perform empirical activity tests for a agent to determine the bioactivity in bioassays and thus determine the effective amount.

20 The present invention also provides a method for inhibiting tumor invasion or metastasis in a subject which comprises administering to the subject a therapeutically effective amount of the aforementioned pharmaceutical composition.

25 One embodiment of the present invention is a composition which comprises a form of soluble RAGE and a pharmaceutically acceptable carrier. In another embodiment the invention provides a composition which comprises the
30 aforementioned agent and a pharmaceutically acceptable carrier.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutically
35 accepted carriers, such as phosphate buffered saline solution, acetate buffered saline solution (a likely vehicle for parenteral administration), water, emulsions such as an oil/water emulsion or a triglyceride emulsion, various types

-17-

of wetting agents, tablets, coated tablets and capsules. An example of an acceptable triglyceride emulsion useful in intravenous and intraperitoneal administration of the compounds is the triglyceride emulsion commercially known as Intralipid®.

When administered orally or topically, such agents and pharmaceutical compositions would be delivered using different carriers. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. The specific carrier would need to be selected based upon the desired method of deliver, e.g., PBS could be used for intravenous or systemic delivery and vegetable fats, creams, salves, ointments or gels may be used for topical delivery.

Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the agent, complexation with metal ions, or incorporation of the agent into or onto particulate preparations of polymeric agents such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, micro emulsions, micelles, unilamellar or multi lamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the agent

-18-

or composition. The choice of compositions will depend on the physical and chemical properties of the agent.

5 The agent of the present invention may be delivered locally via a capsule which allows sustained release of the agent or the peptide over a period of time. Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with
10 polymers (e.g., poloxamers or poloxamines) and the agent coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms
15 protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

20 When administered, agents (such as a peptide comprising the V-domain of sRAGE) are often cleared rapidly from the circulation and may therefore elicit relatively short-lived pharmacological activity. Consequently, frequent injections of relatively large doses of bioactive agents may be required to sustain therapeutic efficacy. Agents modified
25 by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood
30 following intravenous injection than do the corresponding unmodified agents (Abuchowski et al., 1981; Newmark et al., 1982; and Katre et al., 1987). Such modifications may also increase the agent's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical
35 stability of the agent, and greatly reduce the immunogenicity and reactivity of the agent. As a result, the desired *in vivo* biological activity may be achieved by the administration of such polymer-agent adducts less

frequently or in lower doses than with the unmodified agent.

Attachment of polyethylene glycol (PEG) to agents is particularly useful because PEG has very low toxicity in mammals (Carpenter et al., 1971). For example, a PEG adduct of adenosine deaminase was approved in the United States for use in humans for the treatment of severe combined immunodeficiency syndrome. A second advantage afforded by the conjugation of PEG is that of effectively reducing the immunogenicity and antigenicity of heterologous compounds. For example, a PEG adduct of a human protein might be useful for the treatment of disease in other mammalian species without the risk of triggering a severe immune response. The agent of the present invention may be delivered in a microencapsulation device so as to reduce or prevent an host immune response against the agent or against cells which may produce the compound. The agent of the present invention may also be delivered microencapsulated in a membrane, such as a liposome.

Polymers such as PEG may be conveniently attached to one or more reactive amino acid residues in a protein such as the alpha-amino group of the amino terminal amino acid, the epsilon amino groups of lysine side chains, the sulfhydryl groups of cysteine side chains, the carboxyl groups of aspartyl and glutamyl side chains, the alpha-carboxyl group of the carboxy-terminal amino acid, tyrosine side chains, or to activated derivatives of glycosyl chains attached to certain asparagine, serine or threonine residues.

Numerous activated forms of PEG suitable for direct reaction with proteins have been described. Useful PEG reagents for reaction with protein amino groups include active esters of carboxylic acid or carbonate derivatives, particularly those in which the leaving groups are N-hydroxysuccinimide, p-nitrophenol, imidazole or 1-hydroxy-2-nitrobenzene-4-sulfonate. PEG derivatives containing maleimido or haloacetyl groups are useful reagents for the modification

-20-

of protein free sulfhydryl groups. Likewise, PEG reagents containing amino hydrazine or hydrazide groups are useful for reaction with aldehydes generated by periodate oxidation of carbohydrate groups in proteins.

5

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

10

EXPERIMENTAL DETAILSExample 1: Inhibition of Tumor Growth and Spread by Soluble Receptor for Age (sRAGE) in C6-glioma Tumors in Nude Mice

5 An important means by which tumors grow and invade surrounding normal tissue is by a complex series of cell-cell and cell matrix interactions. We have focused on the interaction of tumor cells with matrix-associated
10 components. The Receptor to AGE (RAGE) interacts with a range of physiologically and pathophysiologically-relevant ligands (1-5). In normal developing neurons of the central nervous system, the expression of RAGE is markedly enhanced and co-localizes with that of its ligand, amphoterin.
15 Amphoterin, a matrix-associated polypeptide, is expressed in developing neurons and certain tumor cells, such as rat C6 glioma cells (6-12).

In in vitro experiments, the interaction of neuronal RAGE
20 with amphoterin mediates neurite outgrowth: on amphoterin-coated matrices, neurite outgrowth is inhibited in the presence of either anti-RAGE (Fab)₂ or soluble RAGE (sRAGE; the extracellular two thirds of RAGE). In contrast, blocking access to RAGE had no effect on neurite outgrowth
25 on laminin- or poly-lysine-coated matrices. As in the case of amphoterin, the expression of RAGE decreases after birth in developing rats. Notably, however, the expression of amphoterin is also increased in tumor cells. It has been postulated that in that context, amphoterin may effect
30 matrix degradation by activation of plasminogen: events critical in the ability of tumors to locally invade their immediate environment. Indeed, the ability of tumors to modulate local tissue and vasculature is essential for distal invasion and the development of metastases.

35 In our studies, we tested the hypothesis that blockade of tumor cell RAGE would interfere with the ability of tumors to compromise the integrity of their local environment, at

-22-

least in part by disruption of the RAGE (cellular)-
amphoterin (matrix) interaction. We demonstrate here that
when C6 (rat) glioma cells were transfected with constructs
designed to overexpress soluble RAGE (sRAGE) in the local
5 tumor environment, tumor growth was significantly diminished
in nude mice. In contrast, mock transfection of C6 glioma
cells (vector only) had no effect on tumor growth compared
with untreated cells. Similarly, when C6 glioma cells were
injected into nude mice, intraperitoneal administration of
10 sRAGE resulted in dose-dependent diminution of tumor size.
In contrast, treatment with mouse serum albumin was without
effect.

Taken together, our studies indicate that administration of
15 sRAGE may be an important means by which to limit tumor
growth in the local environment and, likely, the development
of distant metastases. Our studies indicate that
interruption of cell (RAGE)-matrix (amphoterin and/or
similar structures) is at least one mechanism by which sRAGE
20 limits tumor growth. Our data strongly suggest, however,
that sRAGE does not affect the rate of tumor cell growth.

These studies indicate that RAGE may be a novel target for
the prevention of local tumor growth and invasion. Soluble
25 RAGE (sRAGE) may thus represent a model structure for the
development of agents to limit tumor growth and invasion
into the local environment, and, potentially, the
development of distant metastases.

30 MATERIALS AND METHODS:

Cell culture. C6 glioma cells (rat) we obtained from the
American Type Tissue Corporation (ATCC, Rockville, Maryland)
and characterized as previously described (Singh et al,
35 1997). Cells were grown as recommended by the ATCC.
Transfection of constructs expressing either human soluble
RAGE (Neeper et al., 1992) were prepared and transfected
into C6 glioma cells using LipofECTAMINE® (Life

-23-

Technologies, Grand Island, New York). Stable lines were selected using G418 (Life Technologies). Constructs containing vector alone (mock) were transfected into C6 cells as controls.

5

Animal studies. NCR nude mice were obtained from Taconic Laboratories. At the age of 8-10 weeks, C6 glioma cells (usually about 10^5 cells/ml) were injected into the lower backs of the mice. Tumor volume was measured sequentially and recorded. At sacrifice, mice were deeply anesthetized and then tumors removed and weighed.

10

Preparation of mouse soluble RAGE. Murine soluble RAGE was prepared using a baculovirus-Sf9 cell expression system. Murine sRAGE was purified from cellular supernatant using FPLC Mono S[®] (Amersham-Pharmacia[®]) and endotoxin removed using De-Toxigel[®] columns (Pierce, Rockford, Illinois). The final product was pure based on SDS-PAGE electrophoresis (nonreducing conditions). No detectable endotoxin was found in the final samples (limulus amebocyte assay. Sigma Chemical Co., St. Louis, MO). Control mice were treated with mouse serum albumin (MSA: Sigma Chemical Co.)

15

20

Injection of sRAGE into mice. Mice were treated immediately upon injection of the tumor cells into the back of the mouse with the indicated dose of sRAGE or mouse serum albumin (equimolar concentration) through the day of sacrifice. Protein was injected intraperitoneally in a volume of 0.1 ml in phosphate-buffered saline.

25

30

Quantitation of cell number. C6 glioma cells were grown onto the wells of 96-well plates. On day, cells were fixed and relative cell number determined using the CYQUANT[®] assay system from Amersham.

35

RESULTS:

We first determined by RT-PCR that C6 glioma cells expressed

mRNA for RAGE; the presence of RAGE antigen was confirmed by immunohistochemistry. In order to determine if sRAGE might interrupt tumor growth and invasion, we performed two sets of experiments. In the first, C6 glioma cells were transfected with constructs to express either sRAGE or vector alone. In other experiments, C6 glioma cells were injected into nude mice; tumors were generated, and then mice were treated parenterally with either sRAGE or mouse serum albumin.

10

sRAGE-transfected C6 glioma cells.

sRAGE-transfected or mock-transfected C6 glioma cells were injected into nude mice and tumor volume sequentially measured. When tumor volume was measure on days 14, 17 and 21, substantially smaller tumors were observed in sRAGE-transfected cells compared with mock-transfected tumor cells (Fig. 1). On day 14, mean tumor volume in nude mice injected with mock-transfected tumors was $315,276.5 \pm 3,790.4 \mu\text{m}^3$ vs $27,875.4 \pm 4,199.6 \mu\text{m}^3$ mean tumor volume in sRAGE-transfected tumor (11.3-fold difference; $p < 0.00005$). On day 17, mean tumor volume in nude mice injected with mock-transfected tumor was $823,759 \pm 142,841.5 \mu\text{m}^3$ vs. $50,645 \pm 5,109.7 \mu\text{m}^3$ (16.2-fold difference; $p < 0.00005$). On day 21, mean tumor volume in nude mice injected with mock-transfected tumor was $2,200,000 \pm 420,235 \mu\text{m}^3$ vs. $152,000 \pm 22,487.3 \mu\text{m}^3$ (14.5-fold difference; $p = 0.00001$). When evaluated by ELISA, an at least three-fold increase in levels of sRAGE were found in sRAGE-transfected tumors vs. Those transfected with vector alone (mock). Histologic analysis confirmed markedly smaller tumors, with evidence of necrosis.

The growth rate of the transfected tumor cells (sRAGE and mock) derived from analysis of the above in vivo data revealed that there was no difference between the rate of growth of sRAGE-transfected C6 glioma cells compare with those mock-transfected (Fig. 2). When the natural log of

-25-

tumor volume was plotted vs time, the slope of the growth curve for mock-transfected tumor cells the slope of the growth curve was 0.241 ± 0.025 . These data are consistent with the concept that sRAGE does not alter cellular replication. Rather, they suggest that the interaction of tumor cells with their local environment is affected in a manner inconsistent with their local spread and invasion.

To further confirm that transfection of sRAGE into C6 glioma cells did not alter the rate of tumor growth, we demonstrated that when sRAGE-transfected or mock transfected C6 glioma cells were grown in culture, no difference in the cell number was observed at three days incubation (Fig. 3). Using a colormetric assay system from Amersham for quantitation of cell number, on day three, relative cell number of sRAGE-transfected C6 cells was 0.90 ± 0.03 vs. 1.0 ± 0.04 in mock-transfected C6 tumor cells ($p=0.11$). These data were further suggestive of the importance of sRAGE in limiting tumor cell growth and spread into the local tumor environment in vivo and suggest that sRAGE does not impact on cellular replication processes.

Systemic administration of sRAGE.

In order to further delineate the role of sRAGE in limiting tumor growth and invasion into the local environment, C6 tumors were established in NCR nude mice by injection of 10^5 cells/ml (0.1 ml) C6 glioma cells (without transfection). Immediately after injection of tumor cells, intraperitoneal administration of sRAGE was begun (either 20 or 2 $\mu\text{g/day}$) or mouse serum albumin (Fig. 4). On day 14, mean tumor volume in MSA-treated mice was $459,000 \pm 87,457.6 \mu\text{m}^3$. In mice treated with sRAGE, 20 $\mu\text{g/day}$, however, mean tumor volume was decreased 2-fold ($225,000 \pm 38,054 \mu\text{m}^3$; $p=0.03$). In contrast, mice treated with sRAGE, 2 $\mu\text{g/day}$, demonstrated no significant change in tumor volume ($305,000 \pm 79,998$; $p=0.21$ [when compared with those treated with MSA]). On day 17, mean tumor volume in MSA-treated mice was $935,000 \pm 117,499$

-26-

μm^3 . In contrast, mice treated with sRAGE, 2 $\mu\text{g}/\text{day}$, demonstrated no significant change in tumor volume ($637,000 \pm 127,698 \mu\text{m}^3$; $p=0.22$ [when compared with those treated with MSA]).

5

When the growth rate of the tumor cells was plotted, no apparent differences were noted among tumors in mice treated with sRAGE (both doses) or mouse serum albumin (Fig. 5). When the natural log of the tumor volume was plotted vs. time, the slope of the growth curve for C6 glioma tumor cells grown in the presence of mouse serum albumin was 0.264 ± 0.03 . The slopes of the growth curve for C6 glioma cells grown in the presence of sRAGE (20 vs. 2 $\mu\text{g}/\text{ml}$) were not different (0.311 ± 0.04 and 0.313 ± 0.04 , respectively). These data further support the hypothesis that sRAGE does not impact on cellular replication; rather, sRAGE appears to inhibit cellular interaction with matrix components that is necessary for tumor impingement in the local environment.

20 Finally, when C6 cells were cultured in the presence of sRAGE (high and low doses; 20 and 2 $\mu\text{g}/\text{ml}$) or mouse serum albumin, no apparent difference in relative cell number was observed (Fig. 6). Relative cell number (day three) of cells treated with MSA was 1.0 ± 0.045 . Similarly, relative cell number in the presence of sRAGE (20 or 2 $\mu\text{g}/\text{ml}$) was 1.03 ± 0.06 and 1.02 ± 0.06 , respectively. These data further support the concept that sRAGE does not impact upon cellular proliferation; rather, it more likely affects the ability of tumor cells *in vivo* to grow and extend into their local environment.

30

DISCUSSION:

35 A number of factors have been suggested to be important in the ability of tumors to grow and invade their local environment. Central to these involves processes such as angiogenesis, the ability of the tumors to develop tumor-

-27-

specific vasculature; matrix degradation, in which the ability of matrix metalloproteinases (MMPs) to degrade the surrounding matrix is critical for the local spread and invasiveness of tumors; inhibition of the action of tissue inhibitors of metalloproteinases (TIMPs), whose natural function is to inhibit the activity of MMPs; and integrin-cell/integrin/matrix interactions, reactions in which specific ligand-receptor contact initiates signaling cascades that eventuate in tumor spread (14-25).

Certainly, a multitude of factors have been postulated to result in local tumor growth and invasion. In the present studies, the ability of sRAGE to result in diminished tumor volume appears to result not from a direct effect of sRAGE on cellular replication processes. Rather, the interaction of tumor cells with their local environment appears hampered. We postulated that perhaps one means by which this occurs is by inhibition of the tumor RAGE-matrix amphoterin components. Amphoterin and likely related structures have been suggested to activate plasminogen, thereby effecting one means by which, for example, MMPs may be activated in the tumor environment. It is quite possible, however, that additional, as yet unidentified mechanisms exist by which sRAGE exerts beneficial effects in this model.

A current focus of this work is to study the effects of sRAGE in a model of tumor metastases and distal invasion. At this time, studies are underway utilizing Lewis lung carcinoma cells; a model in which distant invasion of the tumor into the pleura and lung parenchyma ensues after removal of the primary lesion (placed onto the lower back of the mice).

In conclusion, while precise mechanisms underlying the beneficial effects of sRAGE are under study in this model of local tumor growth, our data suggest that sRAGE may represent a novel structure in the design of agents to limit

-28-

tumor spread and invasion. In this context, sRAGE may also exert beneficial effects in the prevention of tumor distal growth and metastases.

References for Example 1

1. Schmidt, A-M., et al. J. Biol. Chem. 276: 14987-14997, 1992.
- 5 2. Neeper, M., et al. J. Biol. Chem. 267: 14998-15004, 1992.
3. Hori, O., et al. J. Biol. Chem. 170: 25752-25761, 1995.
4. Yan, S.D., et al. Nature 382: 685-691, 1996.
- 10 5. Yan, S.D., et al. Proc. Natl. Acad. Sci. 94: 5296-5301, 1997.
6. Schmidt, A-M., et al. In Hormones and Signalling, Volume I, Academic Press, p. 41-63, 1997.
7. Rauvala, H. and Pihlaskari, R. J. Biol. Chem. 262: 16625-16635, 1987.
- 15 8. Merenmies, J., et al. J. Biol. Chem. 266: 16722-16729, 1991.
9. Salmivirta, M., et al. Exp. Cell Res. 200: 444-451, 1992.
- 20 10. Mohan, PS, J., et al. Bioc. And Biophyx. Res. Comm. 182: 689-696, 1992.
11. Parkkinen, J., J. Biol. Chem. 268: 19726-19738, 1993.
12. Parkkinen, J. and H. Rauvala. J. Biol. Chem. 266: 16730-16735, 1991.
- 25 13. Singh, S.P., et al. Neuroreport 8: 2359-2363, 1997.
14. Price, J.T., et al. Critical Reviews in Biochemistry and Molecular Biology. 32: 175-253, 1997.
15. Timar, J., et al. Anticancer Research 16(6A): 3299-3306, 1997.
- 30 16. DeVries, TJ, et al. Pathology, Research and Practice 192: 718-733, 1996.
17. Kubota, S., et al. International J. Cancer 70: 106-111, 1997.
18. Powell, WC and LM Matrisian. Current Topics in Microbiology and Immunology 213:1-21, 1996.
- 35 19. Seiki, M. Current Topics in Microbiology and Immunology 213: 23-32, 1996.

-30-

20. Byers SW, CL Sommers, B. Hoxter, AM Mercurio and AJ Tozeren. Cell Science 108: 2053-2064, 1995.
21. Yoneda, J., et al. Experimental Cell Research 217: 169-179, 1995.
- 5 22. Tsang, TC, et al. Cancer Research 54: 882-886, 1994.
23. Grant DS, et al. Pathology, Research and Practice. 190: 854-863, 1994.
24. Spiegel, S., et al. Breast Cancer Research and Treatment 312: 337-348, 1994.
- 10 25. Thorgeirsson, UP, et al. J. Neuro-Oncology 18: 89-103, 1993.

-31-

Example 2 : Amphoterin and Rage: a Novel Interaction
Modulating Tumor Invasion and Migration

SUMMARY:

5

Receptor for AGE (RAGE), a multiligand member of the immunoglobulin superfamily of cell surface molecules, interacts with distinct ligands implicated in development and pathophysiological processes. Previous studies linking RAGE to neurite outgrowth, via ligation of amphoterin, suggested its possible contribution to cellular migration and invasiveness. As high levels of amphoterin and RAGE are present during early neuronal development and in tumors, the impact of the RAGE-amphoterin axis on tumor progression was assessed. Blockade of RAGE-amphoterin interaction potently suppressed primary tumors grown from implanted rat C6 glioma cells, and lung metastases in mice bearing Lewis lung carcinoma. Blockade of RAGE and amphoterin inhibited tumor cell invasion and migration, though proliferation, angiogenesis, matrix attachment and cellular proteolytic (collagenolytic or plasmin) activity were unchanged. These findings highlight a novel paradigm in tumor biology and identify RAGE-amphoterin interaction as an unique target for therapeutic strategies to interrupt both local tumor growth and distant metastasis, whose effects are likely to complement other anti-tumor strategies.

10

15

20

25

INTRODUCTION:

30

35

The Receptor for Advanced Glycation Endproducts (RAGE), a multiligand member of the immunoglobulin superfamily of cell surface molecules (Schmidt et al., 1992; Neeper et al., 1992), interacts with distinct ligands implicated in development and homeostasis (Hori et al., 1995), as well as in certain pathophysiologic situations, such as diabetes, Alzheimer's disease and inflammation (Park et al., 1998; Wautier et al., 1996; Yan et al., 1996; Yan et al., 1997 and

-32-

Hofmann et al., 1998). RAGE was first identified as a signal transduction receptor for products of nonenzymatic glycation and oxidation of proteins/lipids, the Advanced Glycation Endproducts, or AGEs, whose accumulation in disorders such as diabetes has been linked to the pathogenesis of vascular and inflammatory cell complications (Brownlee et al., 1988; and Sell and Monnier, 1989). Subsequent studies identified RAGE as a neuronal/microglial interaction site for amyloid-beta peptide (A β) (Yan et al., 1996; Yan et al., 1997), the proteolytic cleavage product of beta-amyloid precursor protein, whose accumulation in Alzheimer disease brain has been linked to inflammation and neuronal toxicity (Selkoe, 1994; Sisodia and Price, 1995). More recently, we have identified EN-RAGE (Extracellular Novel RAGE binding protein) (Hofmann, et al., 1998), and related members of the S100/calgranulin family of proinflammatory cytokines (Schafer and Heinzmann, 1996; and Zimmer, et al., 1995), as ligands for RAGE. Interaction of EN-RAGEs with RAGE triggers proinflammatory pathways in endothelial cells, macrophages and lymphocytes. Blockade of RAGE suppressed the immune/inflammatory response in murine models of delayed-type hypersensitivity (DTH) and colitis (Hofmann, et al., 1998). In these settings, engagement of RAGE by AGEs, A β , and EN-RAGEs recruits and activates key cell signalling pathways, such as p21^{ras}, MAP kinases, and NF-kB (Yan et al., 1994 and Lander et al., 1997), thereby globally reprogramming cellular functions.

These findings focused attention on RAGE as an integral participant in the cellular response to pathophysiologic stimuli. However, such an immunoglobulin superfamily receptor was unlikely to have evolved solely to mediate interaction with pathologic ligands, suggesting a possible unexplored aspect of the biology of RAGE in development and/or homeostasis. In this context, we previously identified RAGE as a cellular receptor for amphoterin (Hori, et al., 1995), a polypeptide linked to neurite outgrowth in cultured cortical neurons (Rauvala and Pihlaskari, 1987;

-33-

Rauvala et al., 1988; Parkkinen and Rauvala, 1991; and Parkkinen et al., 1993). Engagement of immobilized amphoterin by cell surface neuronal RAGE promoted neurite outgrowth (Hori, et al., 1995). Furthermore, the striking
5 colocalization of RAGE and amphoterin in developing neurons of the central nervous system, especially at the leading edge of advancing neurites, suggested a possible contribution of RAGE-amphoterin interaction to cellular migration and, in other settings, invasiveness. High
10 levels of amphoterin (Parkkinen et al., 1993) and RAGE (see below) in a range of transformed cells highlighted the possibility that amphoterin interaction with RAGE in transformed cells was likely to occur. These considerations led us to hypothesize that amphoterin-mediated activation of
15 RAGE might represent a novel mechanism promoting tumor cell migration and invasion, potentially impacting on local growth and distant spread of malignancies.

RESULTS:

20

RAGE-amphoterin interaction as a mediator of local tumor growth. Rat C6 glioma cells (Benda et al, 1968; Parkkinen et al., 1993) provided an ideal starting point to test our hypothesis. Immunoblotting of C6 glioma cell lysates showed
25 expression of both RAGE (Fig. 7A1, lane 3), as demonstrated by the ≈ 45 kDa band identified with anti-RAGE IgG, and amphoterin, as shown by the ≈ 29 kDa band seen with anti-amphoterin IgG (Fig. 7A2, lane 3). Immunohistochemistry employing nonpermeabilized C6 glioma
30 cells indicated that both RAGE and amphoterin were present on the cell surface.

The extracellular region of RAGE is composed of one "V"-type followed by two "C"-type immunoglobulin-like domains, and
35 comprises the soluble, ligand-bearing portion of the receptor (Neeper et al., 1992). Following the extracellular and hydrophobic transmembrane spanning domains is a short, highly-charged cytosolic tail. Recent studies have shown

-34-

the cytosolic tail of the receptor to be essential for RAGE-dependent intracellular signalling and subsequent cellular activation (Fu et al., 1998; Hofmann et al., 1998). Thus, two strategies for blocking RAGE-mediated cellular stimulation are: 1) expression of a RAGE mutant devoid of the cytosolic tail, producing a truncated form of the receptor present on the cell surface and competent for ligand binding, but one which exerts a dominant-negative effect with respect to RAGE signalling; and, 2) expression of a truncated soluble (s) form of RAGE comprising only the extracellular domain termed sRAGE, which binds up RAGE ligands preventing their access to the cell surface receptor. Stably-transfected clones of C6 glioma cells were produced overexpressing either full-length RAGE (F), the tail-deletion RAGE mutant (T), or sRAGE (S) (Table 1).

-35-

Table 1. Series of stably-transfected clones expressing full-length RAGE (F); cytosolic tail-deletion RAGE (T); soluble RAGE (S); or mock control (vector alone; M).

| 5 | Clone | RAGE Expression, fold increase in densitometry units; F, T or S-transfectants vs M | Natural log tumor volume/day vs time; mean \pm standard error |
|----|-------|--|---|
| | M1 | 1.0 | 0.25 \pm 0.07 |
| 10 | M2 | 1.0 | 0.30 \pm 0.06 |
| | M3 | 1.0 | 0.25 \pm 0.14 |
| | F1 | 11.7 | 0.32 \pm 0.06 |
| | F2 | 9.0 | 0.37 \pm 0.06 |
| 15 | F3 | 22.6 | 0.27 \pm 0.04 |
| | T1 | 20.2 | 0.42 \pm 0.03 |
| | T2 | 7.7 | 0.39 \pm 0.03 |
| | T3 | 9.4 | 0.33 \pm 0.04 |
| 20 | S1 | 3.4 | 0.16 \pm 0.09 |
| | S2 | 7.0 | 0.43 \pm 0.03 |
| | S3 | 8.4 | 0.30 \pm 0.09 |

Compared with mock transfected clones, no significant differences were observed in slope values among F1, F2, or F3 vs M; T1, T2 or T3 vs M; S1, S2, or S3 vs M; in each case, $p > 0.05$.

Three independent clones of each of the rat C6 glioma transfectants, as well as mock-transfected C6 glioma cells (designated M), were implanted into immunocompromised (nude) mice in order to evaluate tumor growth. Compared with mock-transfected tumor cells (Fig. 7B,C), an ≈ 5 -fold increased tumor volume was observed in cells overexpressing full-length RAGE (Fig. 7B,D) (598 ± 113 vs $3,006 \pm 573$ mm³, respectively; $p < 0.001$) (Fig. 7B). Consistent with a role for RAGE signalling in mediating the receptor's effect on tumor behavior, tumor volume was decreased ≈ 3 -fold (196 ± 31 mm³) in tumors derived from C6 glioma cell clones transfected with the tail-deletion mutant (Fig. 7B, E) ($p < 0.01$). Further support for a role of RAGE-mediated cellular activation in modulating tumor cell phenotype was emphasized by the ≈ 6.5 -fold (93 ± 23 mm³) suppression of tumor volume observed in neoplasms derived from sRAGE-transfected C6 glioma cells (Fig. 7B, F) ($p < 0.001$). To be certain that elevated levels of sRAGE in the tumor microenvironment were responsible for its effect on cells, rather than some unusual property of sRAGE produced by the neoplasm itself, soluble receptor was administered once daily to nude mice immediately upon injection of rat C6 glioma cells (Wautier et al., 1996; Park et al., 1998). Systemically administered sRAGE caused dose-dependent suppression of tumor volume (Fig. 7G), though the effect was less dramatic than with tumor mutants producing sRAGE (S1,2,3; Table 1), probably due to higher local concentrations of soluble receptor in sRAGE-transfected tumors.

As amphotericin and RAGE were both present in the tumor bed, a direct way to ascertain their mutual involvement in tumor progression was using monospecific polyclonal antibodies raised to each. Rabbit F(ab')₂ fragments prepared from the antibodies to RAGE and/or amphotericin were administered to immunocompromised (severe combined immunodeficiency, SCID) mice from the time of inoculation with C6 glioma cells. Compared with SCID mice receiving nonimmune F(ab')₂, animals

-37-

treated anti-amphotericin or anti-RAGE F(ab')₂ demonstrated significant reduction in tumor volume after 21 days (4,935 ± 410 vs 2,486 ± 438 mm³ and 2,368 ± 270 mm³, respectively) (Fig. 7H). Furthermore, simultaneous treatment of SCID mice with both anti-RAGE and anti-amphotericin F(ab')₂ resulted in greater reduction in tumor volume compared with animals receiving nonimmune F(ab')₂ (p<0.00001) or either antibody alone. These data substantiated a role for RAGE-amphotericin interaction in modulating tumor progression in a primary tumor model.

Blockade of RAGE suppresses distant metastasis. To ascertain whether RAGE might also contribute to distant tumor spread, the Lewis lung carcinoma model (O'Reilly et al., 1994), in which distant metastases flourish upon removal of primary tumor, was employed. Both RAGE and amphotericin were present in Lewis lung carcinoma cells (Fig. 7A1, lane 2; and Fig. 7A2, lane 2, respectively), and could be localized to the cell surface by immunocytochemistry (not shown). To test our hypotheses, we first prepared stably-transfected Lewis lung carcinoma cells elaborating excess sRAGE. However, following implantation into C57BL/6J mice marked suppression of local tumor growth resulted (data not shown), thereby abrogating the usefulness of this model. As an alternative strategy to block interaction of ligands (amphotericin) with tumor cell RAGE, sRAGE was administered just prior to and after resection of primary tumor. Compared to treatment with vehicle alone, murine serum albumin (MSA), administration of sRAGE resulted in dose-dependent suppression of lung surface metastases (Fig. 8A). Lung surface metastases, observed in vehicle (MSA)-treated mice (8.7 ± 1.4; Fig. 8A, B, F), were virtually undetectable at an sRAGE dose of 100 µg/day (1.0 ± 0.3 and p<0.0001; Fig. 8A, E, G). These findings suggested that blocking access to cell surface RAGE in the presence of excess sRAGE potentially suppressed tumor metastatic capacity.

-38-

Blockade of RAGE and/or amphoterin suppresses invasion and migration of C6 glioma cells: in vitro analyses. It is essential to note that tumors produced by each of the C6 glioma cell transfectants (F, S, and T) demonstrated the same growth rate as tumors from mock-transfected control C6 cells (M), based on the slope of plots of the natural log of tumor volume versus time post-tumor cell inoculation (Table 2). Furthermore, tumor cell proliferation in vitro, comparing wild-type C6 glioma cells with each of the stable transfectants (M, F, T and S), was unchanged (not shown), and incubation of C6 glioma cells with sRAGE was without effect (Fig. 9A). Similarly, no differences were noted in the rate of tumor growth comparing MSA- and sRAGE-treated C6 gliomas, or tumors from animals receiving anti-RAGE or anti-amphoterin F(ab')₂ (Table 2). Thus, as tumor proliferative capacity was not affected, we focussed our attention on other mechanisms, especially RAGE-amphoterin-mediated modulation of tumor cell invasive and migratory properties.

20

-39-

Table 2. Tumor proliferation rate did not differ among mice treated systemically with murine serum albumin (MSA), murine sRAGE or nonimmune/anti-RAGE/anti-amphotericin F(ab')₂ fragments.

| | | | |
|----|---------------------------------------|---|---|
| 5 | Treatment | Natural log tumor volume per day vs time; mean \pm standard error | p value; compared with treatment with MSA or nonimmune F(ab') ₂ |
| 10 | <hr/> | | |
| | MSA | 0.27 \pm 0.03 | - |
| | sRAGE, 2 μ g | 0.31 \pm 0.04 | p>0.1 |
| | sRAGE, 20 μ g | 0.30 \pm 0.04 | P>0.1 |
| 15 | nonimmune F(ab') ₂ | 0.26 \pm 0.02 | - |
| | anti-RAGE F(ab') ₂ | 0.31 \pm 0.03 | p>0.1 |
| | anti-amphotericin F(ab') ₂ | 0.29 \pm 0.04 | p>0.1 |
| | anti-RAGE + | | |
| 20 | anti-amphotericin F(ab') ₂ | 0.35 \pm 0.05 | p>0.1 |

-40-

In vitro tumor cell invasion assays were performed using the Matrigel model. Compared with mock-transfected C6 glioma cells, those bearing full-length RAGE demonstrated enhanced invasion (419 ± 3 vs 550 ± 7 cells; $p < 0.00001$). In contrast, transfected C6 clones bearing the tail-deletion RAGE mutant or overexpressing sRAGE revealed diminished invasion of the Matrigel (265 ± 4 , $p < 0.00001$ vs mock; and 282 ± 7 cells, $p < 0.00001$ vs mock, respectively) (Fig. 9B). Furthermore, C6 glioma cells incubated with sRAGE displayed dose-dependent suppression of tumor cell invasion compared with untreated controls; at the highest dose of sRAGE, $100 \mu\text{g/ml}$, tumor cell invasive capacity was substantially decreased, 462 ± 10 vs 147 ± 8 cells; ($p < 0.00001$; Fig. 9C). Finally, treatment of tumor cells with anti-RAGE and/or anti-amphoterin IgG suppressed C6 cell invasion compared with nonimmune IgG-treated cells (Fig. 9D). These findings suggested that RAGE was an important mediator of tumor cell invasive capacity. Disengagement of RAGE from either its ligand (amphoterin) in the presence of sRAGE, or from intracellular signalling mechanisms with the tail-deletion mutant, inhibited the ability of tumor cells to invade the gel of matrix proteins.

Since cellular migration is an important component of tumor cell invasiveness, the effect of blocking the amphoterin-RAGE axis was investigated in this system. Compared with mock-transfected C6 glioma, those clones expressing full-length RAGE exhibited increased migration (216 ± 3 vs 293 ± 4 cells, respectively; $p < 0.00001$) (Fig. 9E). In contrast, those expressing either tail-deletion RAGE or sRAGE exhibited diminished migration compared with mock-transfected clones (152 ± 3 cells, $p < 0.00001$ vs mock; and 142 ± 3 cells, $p < 0.00001$ vs mock; respectively) (Fig. 9E). Similarly, incubation of C6 glioma cells with sRAGE suppressed migration in a dose-dependent manner. Compared with cells treated with MSA, those treated with the highest dose of sRAGE, $100 \mu\text{g/ml}$, demonstrated a substantial reduction in migration (222 ± 4 vs 106 ± 3 cells,

-41-

respectively; $p < 0.00001$) (Fig. 9F). Additional support for a role of amphotericin-RAGE interaction in mediating C6 glioma migration ability was demonstrated by suppressed cell movement in the presence of anti-RAGE IgG, anti-amphotericin IgG or both antibodies, compared with those treated with nonimmune IgG (Fig. 9G).

Several other mechanisms were considered through which amphotericin engagement of RAGE might suppress tumor growth in vivo, including attachment of tumor cells to the matrix, angiogenesis, and expression of cellular collagenolytic and plasmin activity. Blockade of RAGE in the presence of sRAGE did not affect tumor cell attachment to a Matrigel-coated substrate for up to 60 min after plating (Fig. 10A). At 30 mins after plating, C6 glioma cells treated with MSA, 200 $\mu\text{g/ml}$, revealed $56 \pm 4\%$ attachment; and those treated with sRAGE, 100 $\mu\text{g/ml}$, revealed $50 \pm 3\%$ attached; $p = 0.19$ (Fig. 10A). Similarly, angiogenesis, assessed in the mouse cornea assay was not adversely affected by administration of sRAGE. Basic FGF-induced angiogenesis elicited new capillary growth from the corneal limbus in C57BL/6J mice which was not different whether animals were treated with either MSA or sRAGE (3.1 ± 0.3 vs 2.7 ± 0.2 mm^2 angiogenic area; $p = 0.23$; Fig. 10B). Furthermore, proliferation of cultured HUVEC was not significantly altered in the presence of sRAGE when compared with cells treated with MSA (data not shown). Primary differences in tissue-degradative capacity were also not evident in tumor cells treated with either MSA or sRAGE, as measured by activity of type IV collagenase (Fig. 10C) or plasmin (Fig. 10D).

DISCUSSION:

Molecular mechanisms underlying tumorigenesis comprise a complex mesh of processes promoting unchecked cellular proliferation, invasion and induction of neovessel formation, as well as means of evading the immune response and buttressing metabolic adaptation to nutritional stress.

Upregulation of both amphoterin and RAGE in tumor cells provides an autocrine means for modulating cellular properties in a sustained manner within the neoplasm. Previous studies showed that amphoterin engagement of RAGE
5 on neuron-like cells induced neurite outgrowth and enhanced expression of the receptor via Sp1 sites within the promoter of the RAGE gene (Li et al., 1998; Li and Schmidt, 1997). Furthermore, amphoterin and RAGE have been colocalized to the leading edges of neurites (Parkkinen et al., 1993; Hori
10 et al., 1995). These observations suggested the potential of RAGE-mediated modification of cellular properties to impart invasiveness, and implied that the impact of amphoterin-RAGE interaction might confer an ascending spiral of abnormal cellular behavior; increased expression of
15 amphoterin causing, in turn, enhanced expression of the receptor, thereby further accentuating amphoterin-RAGE-induced changes in cellular properties. Signals regulating RAGE and amphoterin expression might thus contribute importantly to tumor cell phenotype. This
20 situation may prove analogous to tumor expression of type IV collagenase activity (Liotta et al., 1980), Neural Cell Adhesion Molecule-1 (Michalides et al., 1994), CD44 (Ladeda et al., 1998), and certain matrix metalloproteinases (Murray et al., 1996), in which the degree of expression of these
25 mediators has been shown to correlate with tumor invasive and metastatic capacity. The wide distribution of RAGE and amphoterin transcripts in human colon, lung, kidney and other tumors (not shown) suggests that their presence might also underlie basic elements of tumor cell behavior.

30

Amphoterin binding to cell surface RAGE actively recruited signal transduction mechanisms to bring about RAGE-induced changes in cellular phenotype. Transfection of the tail deletion mutants of RAGE in C6 glioma cells, which
35 endogenously expressed low levels of wild-type RAGE, conferred a dominant-negative modulation of cellular properties both *in vivo*, by attenuated growth of primary and metastatic tumors, and *in vitro*, by diminished migration and

-43-

invasion. These observations suggested parallels to the biology of integrins, a family of cell surface transmembrane proteins that participate in tumor attachment, invasion and migration (Hynes, 1992). The intracellular domains of
5 integrins are connected to cytoskeletal proteins and intracellular signal transduction pathways; these domains are postulated to mediate "inside-out" signalling, thereby modulating intercellular communication, cellular differentiation and phenotype (Ginsberg et al., 1992; Heino,
10 1996). Studies are in progress to delineate components of the molecular complex through which the cytosolic domain of RAGE engages signal transduction machinery and triggers effector mechanisms promoting cellular migratory and invasive properties.

15 Amphoterin-RAGE modulation of tumor cell properties is distinct from that of other pathways directly regulating cellular proliferation, angiogenesis, tumor cell-matrix interaction or degradation of critical barrier substrates,
20 such as fibronectin and collagen type IV. In contrast, amphoterin binding to RAGE neither increased the rate of tumor cell growth nor changed tumor cell apoptotic index or p53 expression (Hartwell and Kastan, 1994) (not shown). Blockade of RAGE was without effect on neovessel ingrowth,
25 attachment of tumor cells to matrix elements or tumor cell digestion of basement membrane molecules. Thus, the amphoterin-RAGE axis appeared to exert effects distinct from other well-studied regulators of tumor cell division, neovessel formation, and cell-matrix interactions. These
30 considerations emphasize the role of alternate mechanisms, such as interplay of integrins, laminin receptors, cadherins, cellular adhesion molecules, selectins and/or CD44 in mediating attachment and engagement of structural interfaces in the tumor bed (Price et al., 1997). However,
35 these observations also highlight the possibility that blockade of RAGE may impact on properties of tumor cells in a manner quite distinct from those mentioned above, thereby marking it as a likely complementary target for therapy.

-44-

Conversely, the effect of suppressing amphoterin-RAGE interaction within the tumor bed may augment the effects of other anti-tumor approaches by virtue of their impact on diverse cellular properties. Extrapolation of this concept to the clinical setting suggests that blockade of RAGE might enhance conventional chemotherapy and/or radiotherapy, or newer modalities limiting effective angiogenesis within the enlarging tumor (O'Reilly et al., 1994; Kakeji and Teicher, 1997; Mauceri et al., 1998).

One striking feature of our data is the more effective suppression of tumor cell growth achieved through local expression of sRAGE or dominant-negative RAGE, compared with administration of anti-amphoterin IgG. Although this may reflect, in part, limited penetration of the immune IgG into the tumor bed, it might also suggest the existence of other ligands capable of stimulating RAGE within the tumor itself. In this context, S100/calgranulin proteins, well-known as tumor markers (Cochran et al., 1993; Davies et al., 1993), have been recently identified as RAGE ligands (Hofmann et al., 1998), and may provide another venue for activation of the receptor in the tumor milieu. Taken together, our findings strongly suggest that amphoterin engagement of RAGE modulates potent forces within the tumor and its microenvironment favoring successful local and distant invasion, thereby conferring a survival advantage to primary and metastatic tumors. In contrast, the lack of effect of RAGE blockade on angiogenesis, cellular proliferative rate or tissue-degradative properties assigns amphoterin-RAGE interaction a novel and, potentially, pathophysiologically important role in the modulation of tumor cell behavior.

EXPERIMENTAL PROCEDURES

Cell lines. Rat glial cells (C6), murine Lewis lung carcinoma cells (LLC), and NIH/3T3 cells were purchased from the American Type Culture Corporation (ATCC; Rockville, MD) and cultured according to standard ATCC protocols. Human umbilical vein endothelial cells (HUVEC) were isolated and characterized as previously described (Jaffe et al., 1973) and maintained in Endothelial Cell Growth Medium (EGM; Clonetics, Walkersville MD) according to the manufacturer's instructions.

Development of RAGE constructs. Human RAGE cDNA (Neeper et al., 1992) was inserted into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad CA) behind the human cytomegalovirus major intermediate early promoter/enhancer region by employing enzyme restriction sites KpnI and XhoI. Human cytosolic tail deletion RAGE cDNA was composed of a construct in which the entire cytosolic domain coding sequence was deleted (Neeper et al., 1992) and inserted into pcDNA3 vector by employing EcoRV and XbaI enzyme restriction sites. Human soluble RAGE cDNA was composed of a construct in which both the transmembrane spanning domain and cytosolic domain coding sequences were deleted (Neeper et al., 1992) and inserted into pcDNA3 vector by employing EcoRV and XbaI enzyme restriction sites. All constructs were first transiently-transfected into NIH 3T3 cells and evidence for expression was determined by immunoblotting employing monospecific, polyclonal anti-human RAGE IgG as previously characterized (Schmidt et al., 1992; Hori et al., 1995). This antibody was selectively prepared against soluble human RAGE, therefore, it recognizes the products of all three cDNA constructs.

Development of stably-transfected RAGE tumor cells. C6 rat glioma cells were transfected by employing Lipofectamine (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Cells were selected in the presence of geneticin (G418), 1.5 mg/ml (Life Technologies),

-46-

and individual clones were isolated by means of limiting dilution (Pelicci et al., 1992). Selected clones were tested for RAGE expression by immunoblotting (Schmidt et al., 1992). Four transfected lines were produced as follows: (1) mock transfectants (M), transfected with vector alone but grown and maintained exactly as the RAGE transfectants; (2) full-length RAGE transfectants (F), transfected with construct encoding full-length human RAGE; (3) tail-deletion RAGE transfectants (T), transfected with construct encoding RAGE lacking the cytosolic domain; and (4) soluble RAGE transfectants (S); transfected with construct in which both the transmembrane spanning domain and cytosolic domains were deleted. In each case, three independent clones were established for each transfected cell line.

Production of murine soluble RAGE. The construct encoding murine soluble RAGE was placed into the baculovirus expression system and soluble murine RAGE expressed in *Spodoptera frugiperda* 9 (Sf9) cells (Invitrogen). Murine sRAGE was purified from cellular supernatant as previously described (Park et al., 1998) and devoid of endotoxin prior to intraperitoneal administration to mice by chromatography onto Detox-igel columns (Pierce, Arlington Heights, IL). Absence of endotoxin was documented using a kit from Sigma (St. Louis, MO) (limulus amebocyte assay). Murine serum albumin was purchased from Sigma.

Generation of anti-RAGE and anti-amphoterin F(ab')₂ fragments. Monospecific, polyclonal rabbit anti-human RAGE IgG was prepared and characterized as described. Recombinant rat amphoterin was prepared and characterized as described (Hori et al., 1995). This material was employed to immunize rabbits; serum was then obtained and IgG prepared (Pierce). Nonimmune rabbit serum was purchased from Pocono Rabbit Farms (Canadensis, PA). In all three cases, F(ab')₂ fragments of IgGs were prepared using a kit from Pierce and characterized as per the manufacturer's

-47-

instructions.

Immunoblotting. Immunoblotting of cultured tumor cells was performed by subjecting cells to sonication with a Branson Ultrasonics 250 Sonicator (Branson Ultrasonics, Danbury CT) in buffer containing tris, 0.02M, pH 7.4; NaCl, 0.1 M; PMSF, 0.002M; leupeptin, 0.5 μ g/ml; and pepstatin (0.7 μ g/ml) in the presence of octyl-beta-D-glucopyranoside (1%; Fisher Scientific, Fairlawn, N.J.) at 4°C followed by eight hrs gentle rotation. At the end of that time, cellular extracts were subjected to centrifugation, 11,000xg for 20 mins at 4°C. Supernatant was collected and protein concentration determined using an assay system from Bio-Rad (Hercules CA). Similarly, tumors excised from mice were placed in the same buffer as above, except tissue was subjected to homogenization (Brinkman, Old Westbury, NY). In each case, 30 μ g total protein was subjected to electrophoresis by SDS-PAGE (10% in the case of immunoblotting for detection of RAGE, and 15% in the case of immunoblotting for detection of amphoterin, Novex, San Diego, CA). After electrophoresis, components of the gels were transferred to nitrocellulose membranes (Novex) and subjected to immunoblotting employing rabbit anti-RAGE IgG (25 μ g/ml) or rabbit anti-rat amphoterin IgG (25 μ g/ml) as above. Binding of primary antibody was identified by employing horse radish peroxidase-conjugated goat anti-rabbit IgG (Sigma). In the case of tumor tissue, immunoblotting was performed using anti-p53 IgG (M-19) (Santa Cruz Biotechnology). Binding of primary antibody was identified by employing horse radish peroxidase-conjugated rabbit anti-goat IgG (Sigma). In all cases, binding of secondary antibody was detected using ECL, chemiluminescence kit from Amersham Pharmacia Biotech (Piscataway, N.J.) according to the manufacturer's instructions. For sRAGE-generating clones, transfected tumor cells were plated onto tissue culture-treated plastic dishes containing 6 wells for 12 hrs in complete medium. At the end of that time, cells were washed and then incubated in medium without FBS for 24 hrs. Supernatant was then

-48-

collected and subjected to SDS PAGE/immunoblotting as above. Densitometric analysis of band intensity was performed using Image Quant (Molecular Dynamics, Foster City, CA).

- 5 **Assessment of apoptosis in C6 glioma tumor.** At time of sacrifice, C6 glioma tumors were removed from nude mice and fixed in formalin (10%). Paraffin-embedded tissue sections were prepared, each 5 μ m thick. Assessment of apoptosis was made by TUNEL assay employing the In Situ Cell Death
10 Detection kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions.

Model of local tumor growth: effect of soluble RAGE or RAGE-transfection. Rat C6 glioma cells (wild-type or cloned
15 transfectants as above), 1×10^5 /0.1 ml PBS, were injected subcutaneously into the dorsal midline of female NCR immunocompromised (nude) mice, age 4-6 weeks (Taconic Farms, Germantown, NY). Where indicated, immediately after injection of tumor cells, mice received daily
20 intraperitoneal injections of protein vehicle murine serum albumin (MSA), or the indicated concentration of murine sRAGE. Tumors were measured on days 14, 17 and 21 with calipers and tumor volume was calculated as follows: $V = \frac{1}{6} \times h(h^2 + 3a^2)$, where h=height of the tumor segment; a=
25 (length + width of the tumor)/4; and V=volume of the tumor (Weast, 1966).

Model of local tumor growth: effect of blockade of amphoterin and/or RAGE. Wild-type rat C6 glioma cells were
30 injected into the dorsal midline of female mice with severe combined immunodeficiency (SCID; Taconic Farms). The mice were divided into four groups, each receiving intraperitoneal injection of the indicated $F(ab')_2$ fragment every three days: the first received nonimmune rabbit $F(ab')_2$
35 fragments (400 μ g/dose); the second received rabbit anti-RAGE $F(ab')_2$ fragments (200 μ g/dose); the third received rabbit anti-amphoterin $F(ab')_2$ fragments (200 μ g/dose); and the fourth group received rabbit anti-RAGE $F(ab')_2$ fragments

-49-

(200 μ g/dose) + anti-amphotericin F(ab')₂ fragments (200 μ g/dose). Tumors were measured and all mice sacrificed on day 21.

5 **Model of metastatic tumor growth and invasion.** A model of tumor metastasis was established according to previously-published procedures (O'Reilly et al., 1994). Cultured Lewis lung murine carcinoma cells (2×10^6 in 0.1 ml PBS) were injected into the dorsal midline of male, 6-8 week
10 old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). Primary tumors were surgically excised when tumor volume reached 1,500 mm³ (usually on day 14). Three days prior to removal of primary tumor, mice received the indicated dose of sRAGE or MSA once daily by intraperitoneal injection.
15 Treatment was continued until sacrifice, 21 days after removal of primary tumor. At sacrifice, numbers of lung surface metastases were counted under 4x-magnification using an Olympus microscope (Melville NY) upon intratracheal injection of India Ink (15%).

20 **Murine corneal angiogenesis assay.** To determine if administration of murine soluble RAGE altered angiogenesis, a corneal micropocket angiogenesis assay was performed as described (O'Reilly et al., 1994). Briefly, pellets
25 containing basic fibroblast growth factor (bFGF; Intergen, Purchase NY) and sucralfate (Bukh Meditec, Denmark) were introduced into corneal pockets of male, 6-8 week old C57BL/6J mice. Mice received either intraperitoneal injection of murine serum albumin or murine soluble RAGE.
30 Mice were sacrificed after 5 days treatment, and the eyes were examined under slit-lamp biomicroscopy (Nikon FS-2, Melville NY) for angiogenic response. Area of angiogenic response was calculated by employing the formula for an ellipse: $A = [(CH \times 0.4) \times VL \text{ (mm)} \times \pi] / 2$, where CH = clock
35 hours with a 360° reticule (30° equals one clock hour) and VL = maximal vessel length.

-50-

Cell proliferation assay. Proliferation of cultured HUVEC or C6 glioma cells was performed on 96-well tissue culture-treated wells (Corning, Corning NY). Initial cell density at plating was 1×10^3 cells/well. Cells were incubated for 3 hours in standard medium to allow attachment. At the end of that time, the indicated concentrations of either MSA or sRAGE were added; medium was changed daily and fresh sRAGE or MSA added. Three days after plating, cell number was assessed using the CyQUANT Cell Proliferation Assay kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Absence of untoward cell death was documented in both conditions by exclusion of trypan blue (Sigma).

In vitro migration and invasion assays. Assessment of in vitro tumor cell migration was performed as previously described (Valente et al., 1998). Briefly, wild-type tumor cells as indicated (3×10^6) were added to the upper chambers of Transwell 6-well plates (Costar, Cambridge MA), 8 μ m pore-sized filter. The chemoattractant, conditioned medium from NIH 3T3 cells, was added to the lower chamber. Cells were incubated with the indicated concentrations of either MSA or sRAGE for 5 hrs at 37°C in an incubator containing CO₂ (5%). In other experiments, tumor cells were pretreated with the indicated concentration of nonimmune IgG, anti-RAGE IgG, anti-amphotericin IgG or anti-RAGE + anti-amphotericin IgG for one hr at 4°C prior to placement in the Transwell chambers. In both cases, after 5 hrs, cells remaining on the upper side of the filter were gently-removed by cotton swabbing. The bottom side of the filter was then fixed in methanol and stained with Giemsa (Sigma). Cells in 10 hpf were then counted. Migration assays on each of the four groups of transfected clones was performed similarly. Invasion assays were performed in an identical manner except that the upper chambers of the Transwell culture dishes were pretreated with Matrigel, 500 μ g/filter (Becton Dickinson, Bedford MA) as previously described (Albini et al., 1987).

-51-

In vitro attachment assays. 96-well tissue culture treated-dishes (Corning) were coated with Matrigel (30 μ g/well). To each well were then added rat C6 glioma cells (1×10^4 cells per well) in the presence of sRAGE or MSA. At the indicated times, wells were depleted of medium by aspiration and washed twice with PBS (Life Technologies). At those time points, cell number per well was quantified using the CyQUANT assay kit.

Assessment of type IV collagenase activity. Assessment of activity of type IV collagenase was performed as previously described (Nakajima et al., 1987). Briefly, N-[propionate-2,3- 3 H]-propionylated human type IV collagen (NEN Life Science, Boston MA) was admixed with unlabelled human type IV collagen (Life Technologies) and adjusted to 100,000 cpm/200 μ g/ml in acetic acid (0.5M). Aliquots containing 3,000 cpm of the type IV collagen were placed into the wells of 96 well tissue culture-treated dishes and dried overnight in a laminar air flow hood. Rat C6 glioma cells (2×10^5 in 0.2 ml medium) were placed onto the dried 3 H-collagen film and incubated at 37°C in a humidified atmosphere (CO₂, 5%). Cells were treated with either MSA (200 μ g/ml) or sRAGE (100 μ g/ml). At 1, 3, 6, 12 or 24 hrs, the culture supernatant was removed and mixed with solution containing ice-cold trichloroacetic acid (10%) and tannic acid (0.5%) (0.05 ml). After 30 mins incubation at 4°C, the mixture was subjected to centrifugation at 10,000xg for 10 mins at 4°C to precipitate undigested material. The radioactivity of the supernatant was measured in a liquid scintillation counter (LKB, Gaithersburg, MD).

Assessment of plasmin activity. Rat C6 glioma cells (2×10^5 cells/0.2 ml phenol-free medium) were placed into 96 well tissue culture-treated dishes. Cells were incubated in the presence of MSA (200 μ g/ml) or sRAGE (100 μ g/ml) in medium containing Spectrozyme PL (0.5M, American Diagnostica, Greenwich CT). At 1, 3, 6, 12 or 24 hrs, the plates were placed into a Bio Kinetics Reader (Bio Tek Instruments,

-52-

Winooski VT) and A_{405nm} recorded.

Analysis of RAGE and amphoterin expression in human tumors.

RNA derived from human tumors was generously provided by Dr. Benjamin Tycko, Columbia University Tumor Bank, Department
5 of Pathology. Five micrograms of RNA were reverse
transcribed with Superscript II (Life Technologies),
according to the manufacturer's protocol. The following
primers were employed for detection of transcripts for RAGE
(5'-ACACTGCAGTCGGAGCTAAT-3' (Seq. ID No. 7) and
10 5'-AAGATGACCCCAATGAGCAG-3' (Seq. ID No. 8)) and for
detection of amphoterin (5'-AAGAAGTGCTCAGAGAGGTG-3' (Seq. ID
No. 9) and 5'-TAAGGCTGCTTGTCATCTGC-3' (Seq. ID No. 10)).
Conditions for PCR consisted of 40 amplification cycles
employing 62°C as the annealing temperature.

15

Statistical analysis. Statistical comparisons among groups
was determined using one-way analysis of variance (ANOVA);
where indicated, individual comparisons were performed using
students' t-test. Statistical analyses comparing the slopes
20 of tumor growth rate curves were performed using a program
in Microsoft Excel (Redmond WA). Statistical significance
was ascribed to the data when $p < 0.05$.

References for Example 2

- Albini, A., Iwamoto, Y., Kleinman, H.K., Martin, G.R., Aaronson, S.A., Kozlowski, J.M., and McEwan, R.N. (1987) A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res.* 47, 3239-3245.
- Benda, P., Lightbody, J., Sato, G., Levine, L., and Sweet, W. (1968) Differentiated rat glial cell strain in tissue culture. *Science* 161, 370-371.
- Brownlee, M., Cerami, A., and Vlassara, H. (1988) Advanced glycosylation endproducts in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.* 318, 1315-1320.
- Cochran, A.J., Lu, H.F., Li, P.X., Saxton, R., and Wen, D.R. (1993) S-100 protein remains a practical marker for melanocytic and other tumors. *Melanoma Res.* 3, 325-330.
- Davies, B.R., Davies, M.P., Gibbs, F.E., Barraclough, R., and Rudland, P.S. (1993) Induction of the metastatic phenotype by transfection of a benign rat mammary epithelial cell line with the gene for p9Ka, a rat calcium-binding protein, but not with the oncogene EJ-ras-1. *Oncogene* 8, 999-1008.
- Fu, C., Pischetsrieder, M., Hofmann, M., Yan, S.F., Stern, D., Schmidt, A.M. (1998).
- Carboxymethyl-lysine AGE modifications of proteins are ligands for RAGE that activate cell signalling pathways. *Circ. Suppl.* 98, # 1651.
- Ginsberg, M.H., Du, X., and Plow, E.F. (1992) Inside-out integrin signalling. *Curr. Opin. Cell. Biol.* 4, 766-771.
- Hartwell, L.H., and Kastan, M.B. (1994) Cell cycle control

-54-

and cancer. Science 266, 1821-1828.

Heino, J. (1996) Biology of tumor cell invasion: interplay of cell adhesion and matrix degradation. Int. J. Cancer 65,
5 717-722.

Hofmann, M., Drury, S., Fu, C., Li, J., Qu, X., Qu, W., and Schmidt, A.M. (1998) EN-RAGE (Extracellular Novel RAGE binding protein) activates endothelial cells and macrophages
10 to mediate inflammatory responses. Circ. (Suppl). 98, #1657.

Hori, O., Brett, J., Slattery, T., Cao, R., Zhang, J., Chen, J., Nagashima, M., Nitecki, D., Morser, J., Stern, D., and Schmidt, A.M. (1995). The receptor for advanced glycation
15 endproducts (RAGE) is a cellular binding site for amphotericin: mediation of neurite outgrowth and coexpression of RAGE and amphotericin in the developing nervous system. J.Biol.Chem. 270, 25752-25761.

20 Hynes, R.O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69, 11-25.

Jaffe, E., Nachman, R., Becker, C., and Minick, R. (1973)
25 Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. 52,2745-2756.

Kakeji, Y., and Teicher, B.A. (1997) Preclinical studies of
30 the combination of angiogenic inhibitors with cytotoxic agents. Invest. New Drugs 15, 39-48.

Ladeda, V., Aguirre Ghiso, J.A., and de Kier Joffe, E.B. (1998) Function and expression of CD44 during spreading,
35 migration and invasion of murine carcinoma cells. Exp. Cell Res. 242, 515-527.

Lander, H.L., Tauras, J.M., Ogiste, J.S., Moss, R.A., and

-55-

- A.M. Schmidt. (1997) Activation of the Receptor for Advanced Glycation Endproducts triggers a MAP Kinase pathway regulated by oxidant stress. J. Biol. Chem. 272,17810-17814.
- 5 Li, J., Qu, X., and A.M. Schmidt. (1998) Sp1 binding elements in the promoter of RAGE are essential for amphoterin-mediated gene expression in cultured neuroblastoma cells. J. Biol. Chemistry 273, 30870-30878.
- 10 Li, J., and Schmidt, A.M. (1997). Characterization and functional analysis of the promotor of RAGE, the receptor for advanced glycation endproducts. J. Biol. Chem. 271, 16498-16506.
- 15 Liotta, L.A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C.M., Shafie, S. (1980) Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 284, 67-68.
- 20 Mauceri, H.J., Hanna, N.N., Beckett, M.A., Gorski, D.H., Staba, M.-J., Stellato, K.A., Bigelow, K., Heimann, R., Gately, S., Dhanabal, M., Soff, G.A., Sukhatme, V.P., Kufe, D.W., and Weichselbaum, R.R. (1998) Combined effects of angiostatin and ionizing radiation in antitumor therapy.
- 25 Nature 394, 2887-291.
- Michalides, R., Kwa, B., Springall, D., Van Zandwijk, N., Koopman, J., Hilkens, J., and Mooi, W. (1994) NCAM and lung cancer. Int. J. Cancer 8, 34-37.
- 30 Murray, G.I., Duncan, M.E., O'Neil, P., Melvin, W.T., and Fothergill, J.E. (1996) Matrix metalloproteinase-1 is associated with poor prognosis in colorectal cancer. Nat. Med. 2, 461-462.
- 35 Nakajima, M., Welch, D.R., Belloni, P.N., and Nocolson, G.L. (1987) Degradation of basement membrane type IV collagen and lung subendothelial matrix by rat mammary adenocarcinoma

-56-

cell clones of differing metastatic potentials. Cancer Res. 47, 4869-4876.

- 5 Neeper, M., Schmidt, A.M., Brett, J., Yan, S.D., Wang, F., Pan, Y.C., Elliston, K., Stern, D., and Shaw, A. (1992). Cloning and expression of RAGE: a cell surface receptor for advanced glycosylation end products of proteins. J. Biol.Chem. 267, 14998-15004.
- 10 O'Reilly, M.S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R.A., Moses, M., Lane, W.S., Cao, Y., Sage, E.H., and Folkman, J. (1994) Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79, 315-328.
- 15 Park, L., Raman, K.G., Lee, K.J., Lu, Y., Ferran, L.J., Chow, W.S., Stern, D., and Schmidt, A.M. (1998) Suppression of accelerated diabetic atherosclerosis by soluble Receptor for AGE (sRAGE). Nature Medicine 4,1025-1031.
- 20 Parkkinen, J., Rauilo, E., Merenmies, J., Nolo, R., Kagander, E.O., Baumann, M., and Rauvala, H. (1993) Amphoterin, the 30-kDa protein in a family of HMG1-type polypeptides. Enhanced expression in transformed cells, leading edge localization, and interactions with plasminogen activation.
- 25 J. Biol. Chem. 268,19726-19738.
- Parkkinen, J. and Rauvala, H. (1991) Interactions of plasminogen and tissue plasminogen activator (t-PA) with
- 30 amphoterin. J. Biol. Chem. 266, 16730-16735.
- Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T., and Pelicci, P.G. (1992) A novel transforming protein
- 35 (SHC) with an SH2 domain is implicated in mitogenic signal transduction. Cell 70, 93-104.
- Price, J.T., Bonovich, M.T., and Kohn, E.C. (1997) The

biochemistry of cancer dissemination. Critical Rev. in Biochem. and Molecular Biol. 32, 175-253.

5 Rauvala, H., Merenmies, J., Pihlaskari, R., Korkolainen, M.,
Huhtala, M.-L., and Panula, P. (1988) The adhesive and
neurite-promoting molecule p30: analysis of the
amino-terminal sequence and production of antipeptide
antibodies that detect p30 at the surface of neuroblastoma
cells and of brain neurons. J. Cell. Biol. 107, 2293-22305.

10

Rauvala, H., and Pihlaskari, R. (1987) Isolation and some
characteristics of an adhesive factor of brain that enhances
neurite outgrowth in central neurons. J. Biol. Chem.
262, 16625-16635.

15 Schafer, B.W., and Heinzmann, C.W. (1996). The S100 family
of EF-hand calcium-binding proteins: functions and
pathology. TIBS 21, 134-140.

20 Schmidt, A.M., Vianna, M., Gerlach, M., Brett, J., Ryan, J.,
Kao, J., Esposito, C., Hegarty, H., Hurley, W., Clauss, M.,
Wang, F., Pan, Y.C., Tsang, T.C., and Stern, D. (1992)
Isolation and characterization of binding proteins for
advanced glycosylation endproducts from lung tissue which
are present on the endothelial cell surface. J. Biol. Chem.
25 267, 14987-14997.

30 Sell, D., and Monnier, V. (1989) Structure elucidation of a
senescence cross-link from human extracellular matrix;
implication of pentoses in the aging process. J. Biol. Chem.
264, 21597-21602.

35 Selkoe, D. (1994) Alzheimer's disease: a central role for
amyloid. J. Neuropathol. Exp. Neurol 53, 438-447.

40 Sisodia, S., and Price, D. (1995) Role of the beta-amyloid
protein in Alzheimer's disease. FASEB J. 9, 366-370.

Valente, P., Fassina, G., Melchior, A., Masiello, L., Cilli,

-58-

M., Vacca, A., Onisto, M., Santi, L., Stetler-Stevenson, W.G., and Albini, A. (1998) TIMP-2 over-expression reduces invasion and angiogenesis and protects B16F10 melanoma cells from apoptosis. *Int. J. Cancer* 75, 246-253.

5

Wautier, J-L., Zoukourian, C., Chappey, O., Wautier, M.P., Guillausseau, P.J., Cao, R., Hori, O., Stern, D., and Schmidt, A.M. (1996) Receptor-mediated endothelial cell dysfunction in diabetic vasculopathy: soluble receptor for advanced glycation endproducts blocks hyperpermeability. *J. Clin. Invest.* 97,238-243.

10

Weast, R. (1966) Handbook of Chemistry and Physics. The Chemical Rubber Company, Cleveland, Ohio.

15

Yan, S.D., Chen, X., Fu, J., Chen, M., Zhu, H., Roher, A., Slattey, T., Nagashima, M., Morser, J., Migheli, A., Nawroth, P., Godman, G., Stern, D., and Schmidt, A.M. (1996). RAGE and amyloid beta peptide neurotoxicity in Alzheimer's disease. *Nature* 382, 685-691.

20

Yan, S-D., Schmidt A-M., Anderson, G., Zhang, J., Brett, J., Zou, Y-S., Pinsky, D., and Stern, D. (1994) Enhanced cellular oxidant stress by the interaction of advanced glycation endproducts with their receptors/binding proteins. *J. Biol. Chem.* 269,9889-9897.

25

Yan, S.D., Zhu, H., Fu, J., Yan, S.F., Roher, A., Tourtellotte, W., Rajavashisth, T., Chen, X., Stern, D., and Schmidt A.M. (1997). Amyloid-beta peptide-RAGE interaction elicits neuronal expression of M-CSF: a proinflammatory pathway in Alzheimer's disease. *Proc.Natl.Acad.Sci.* 94, 5296-5301.

30

Zimmer, D.B., Cornwall, E.H., Landar, A., and Song, W. (1995). The S100 protein family: history, function, and expression. *Brain Research Bulletin* 37, 417-429.

35

-59-

What is claimed is:

1. A method for inhibiting tumor invasion or metastasis in a subject which comprises administering to the
5 subject a therapeutically effective amount of a form of soluble Receptor for Advanced Glycation Endproducts (RAGE).
2. The method of claim 1, wherein the form of soluble
10 RAGE comprises a peptide having the sequence from alanine at position 1 to serine at position 332 of human RAGE.
3. The method of claim 1, wherein the form of soluble
15 RAGE comprises a peptide having the sequence from methionine at position 1 to isoleucine at position 120 of human RAGE.
4. The method of claim 1, wherein the form of soluble
20 RAGE is a peptide expressed by a replicable vector containing nucleic acid encoding the form of soluble RAGE.
5. The method of claim 4, wherein the replicable vector
25 is capable of expressing the peptide within a tumor cell in a subject.
6. The method of claim 5, wherein the tumor cell is a
eukaryotic cell.
7. The method of claim 4, wherein the replicable vector
30 is a plasmid, an attenuated virus, a phage, a phagemid or a linear nucleic acid.
8. The method of claim 1, further comprising
35 administering a pharmaceutically acceptable carrier to the subject during the administration of the form of soluble RAGE.

-60-

9. The method of claim 1, wherein the administration is via intralesional, intraperitoneal, intramuscular or intravenous injection; infusion; intrathecal administration; subcutaneous administration; liposome-mediated delivery; or topical, nasal, oral, ocular or otic delivery.
10. The method of claim 1, wherein the form of soluble RAGE comprises a V domain of naturally occurring soluble RAGE.
11. The method of claim 1, wherein the form of soluble RAGE comprises a C domain of naturally occurring soluble RAGE.
12. The method of claim 1, wherein the subject is a mammal.
13. The method of claim 12, wherein the mammal is a human.
14. The method of claim 1, wherein the form of soluble RAGE is administered daily, weekly or monthly.
15. The method of claim 1, wherein the therapeutically effective amount comprises a dose from about 0.000001 mg/kg body weight to about 100 mg/kg body weight.
16. The method of claim 1, wherein the therapeutically effective amount comprises a dose of from about 100 ng/day/kg body weight to about 200 mg/day/kg body weight.
17. A method for evaluating the ability of an agent to inhibit tumor invasion in a local cellular environment which comprises:
- (a) admixing with cell culture media an effective amount of the agent;

-61-

- (b) contacting a tumor cell in cell culture with the media from step (a);
- (c) determining the amount of spreading of the tumor cell culture, and
- (d) comparing the amount of spreading of the tumor cell culture determined in step (c) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit tumor invasion in the local cellular environment.
18. The method of claim 17, wherein the tumor cell is a eukaryotic cell.
19. The method of claim 17, wherein the tumor cell is a cell of a subject.
20. The method of claim 19, wherein the subject is a human, a mouse, a rat, a dog or a non-human primate.
21. The method of claim 17, wherein the agent comprises a peptide, a peptidomimetic, a nucleic acid, a synthetic organic molecule, an inorganic molecule, a carbohydrate, a lipid, an antibody or fragment thereof, or a small molecule.
22. The method of claim 21, wherein the antibody is a monoclonal antibody.
23. The method of claim 21, wherein the antibody is a polyclonal antibody.
24. The method of claim 21, wherein the fragment of the antibody comprises a Fab fragment.
25. The method of claim 21, wherein the fragment of the antibody comprises a complementarity determining

-62-

region or a variable region.

26. The method of claim 21, wherein the peptide is a synthetic peptide or a peptide analog.
- 5 27. The method of claim 21, wherein the peptide comprises at least a portion of the sequence -Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-10 Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val- (Seq. I.D. No. 3).
28. The method of claim 21, wherein the peptide comprises at least a portion of the sequence -Gly-Ser-Asn-Lys-15 Gly-Ala-Ile-Ile-Gly-Leu-Met- (Seq. I.D. No. 4).
29. The method of claim 21, wherein the peptide has the amino acid sequence A-Q-N-I-T-A-R-I-G-E-P-L-V-L-K-C-K-G-A-P-K-K-P-P-Q-R-L-E-W-K (Seq. I.D. No. 5).
- 20 30. The method of claim 21, wherein the peptide has the amino acid sequence A-Q-N-I-T-A-R-I-G-E (Seq. I.D. No. 6).
- 25 31. The method of claim 21, wherein the agent is a form of soluble human RAGE.
32. The method of claim 21, wherein the agent is an extracellular portion of human RAGE.
- 30 33. The method of claim 21, wherein the agent inhibits an interaction between the tumor cell and an extracellular matrix molecule.
- 35 34. The method of claim 21, wherein the extracellular matrix molecule is a laminin, a fibronectin, amphoterin, a cadherin, an integrin or a hyaluronic acid.

-63-

35. The method of claim 34, wherein the integrin is an $\alpha V\beta V$ integrin, an $\alpha V\beta III$ integrin, or an $\alpha I\beta II$ integrin.
- 5 36. The method of claim 21, wherein the agent inhibits binding of RAGE to amphoterin.
37. The method of claim 21 wherein the agent binds to RAGE.
- 10 38. The method of claim 21 wherein the agent binds to amphoterin.
- 15 39. A pharmaceutical composition which comprises a therapeutically effective amount of the agent evaluated in claim 17 and a pharmaceutically acceptable carrier.
- 20 40. The pharmaceutical composition of claim 39, wherein the carrier is a diluent, an aerosol, a topical carrier, an aqueous solution, a replicable nucleic acid vector, a liposome, a magnetic bead, a nonaqueous solution or a solid carrier.
- 25 41. A method for inhibiting tumor invasion or metastasis in a subject which comprises administering to the subject a therapeutically effective amount of the pharmaceutical composition of claim 39.

1/15

FIG. 1A

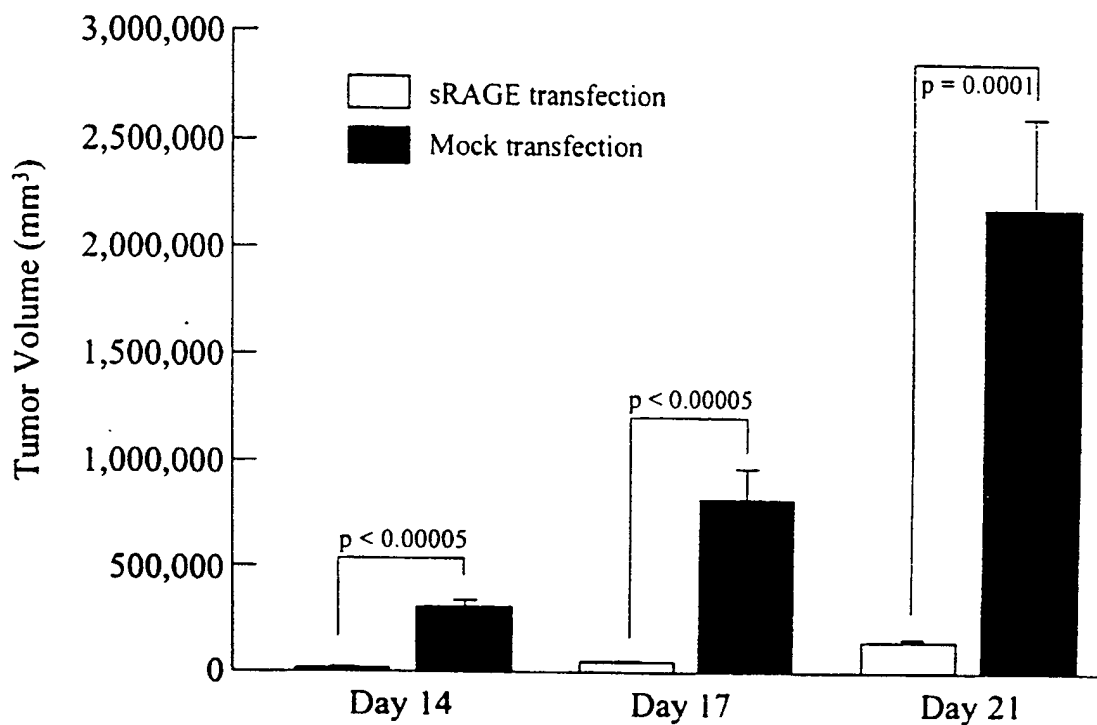
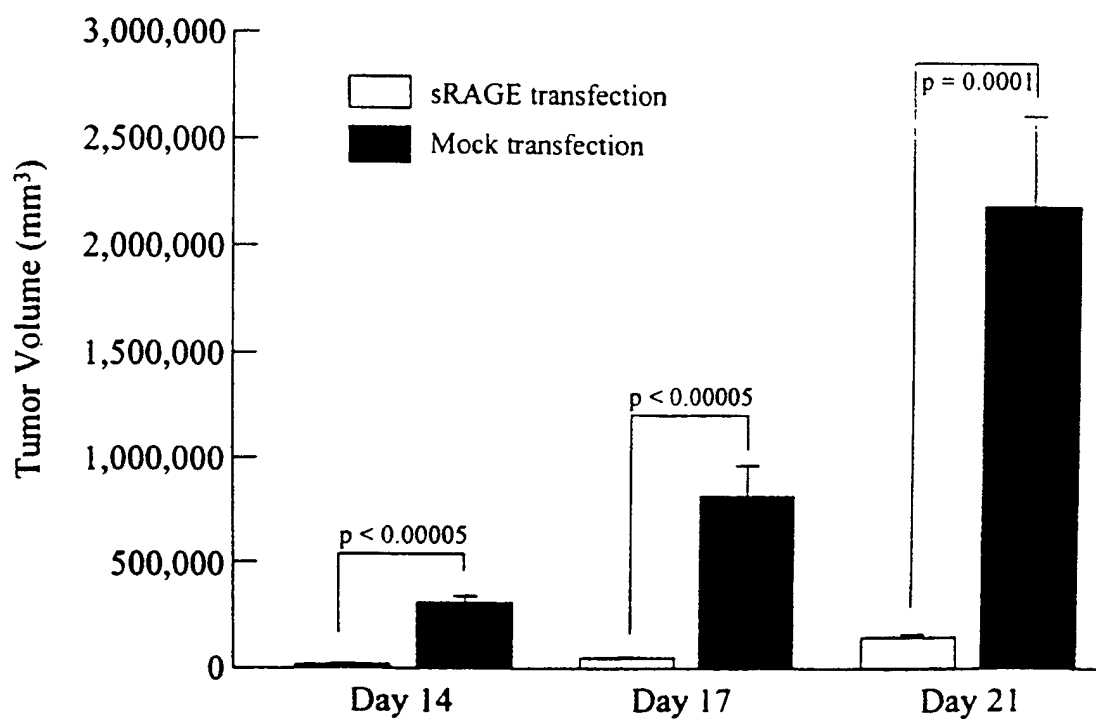


FIG. 1B



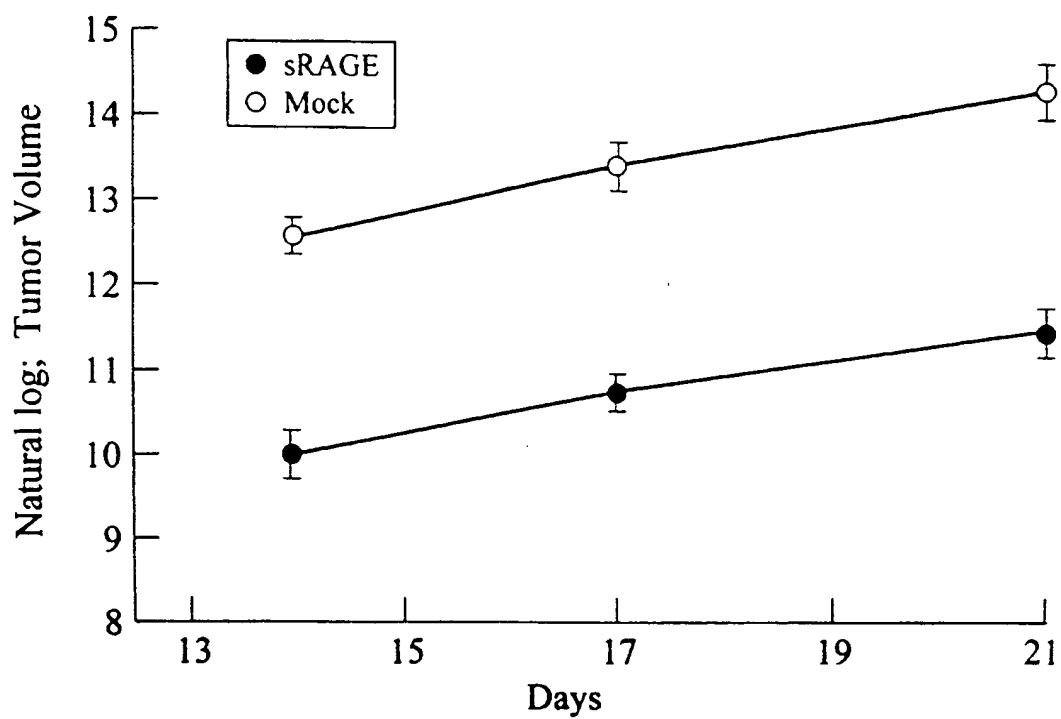
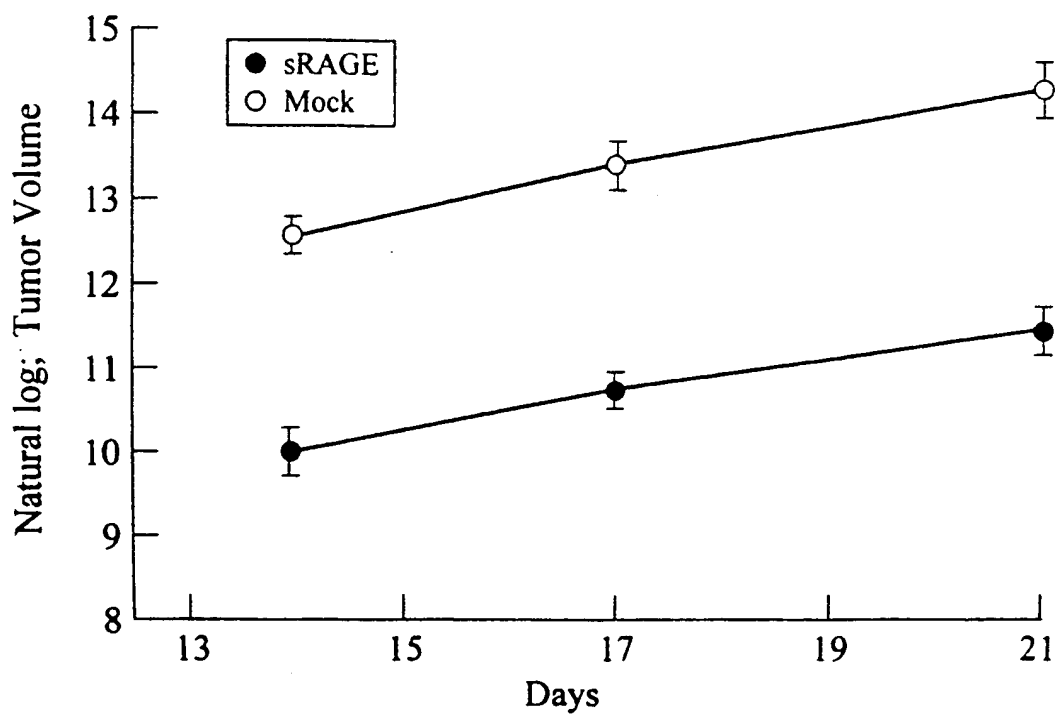
2/15
FIG. 2A

FIG. 2B



3/15

FIG. 3A

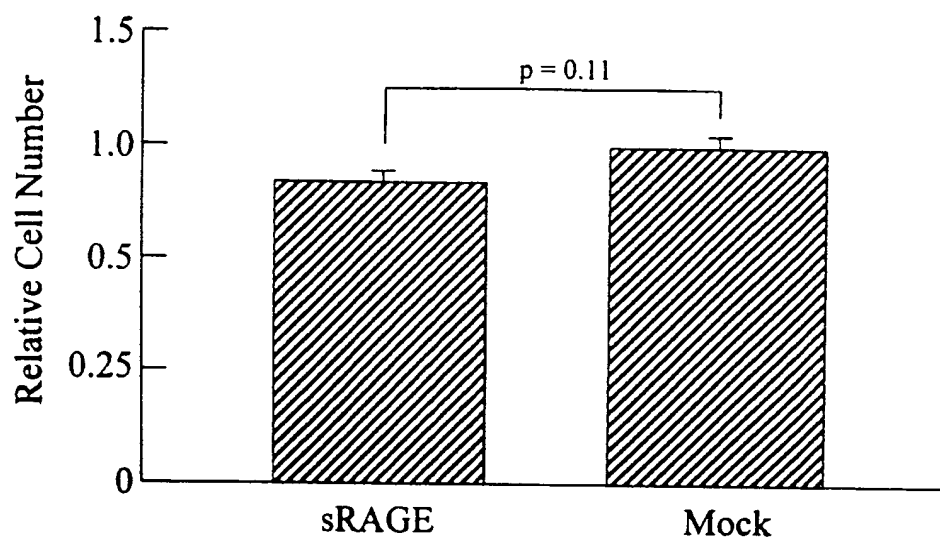
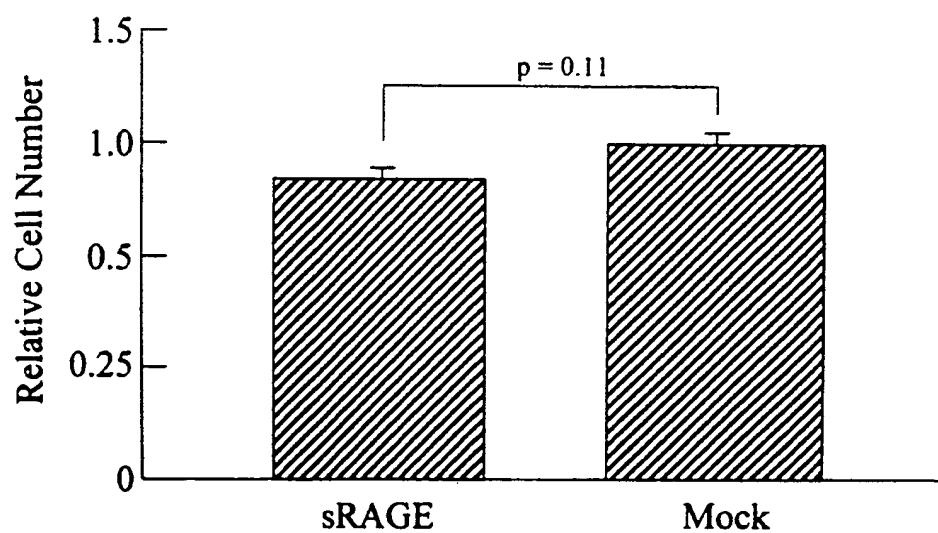


FIG. 3B



4/15

FIG. 4A

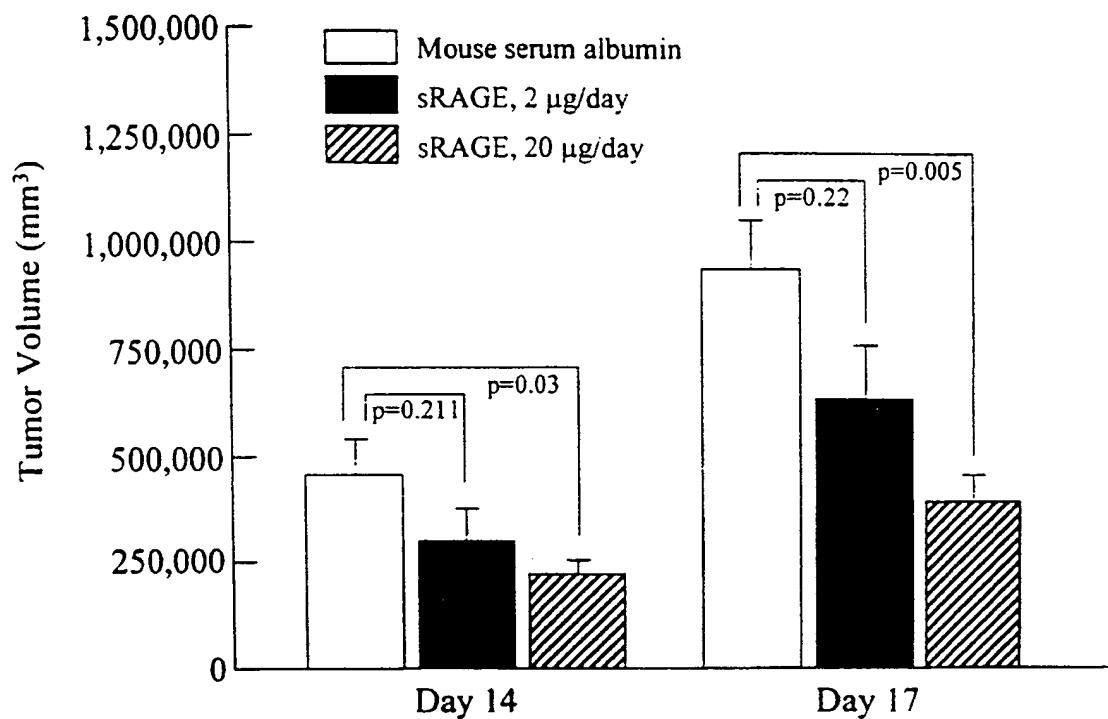
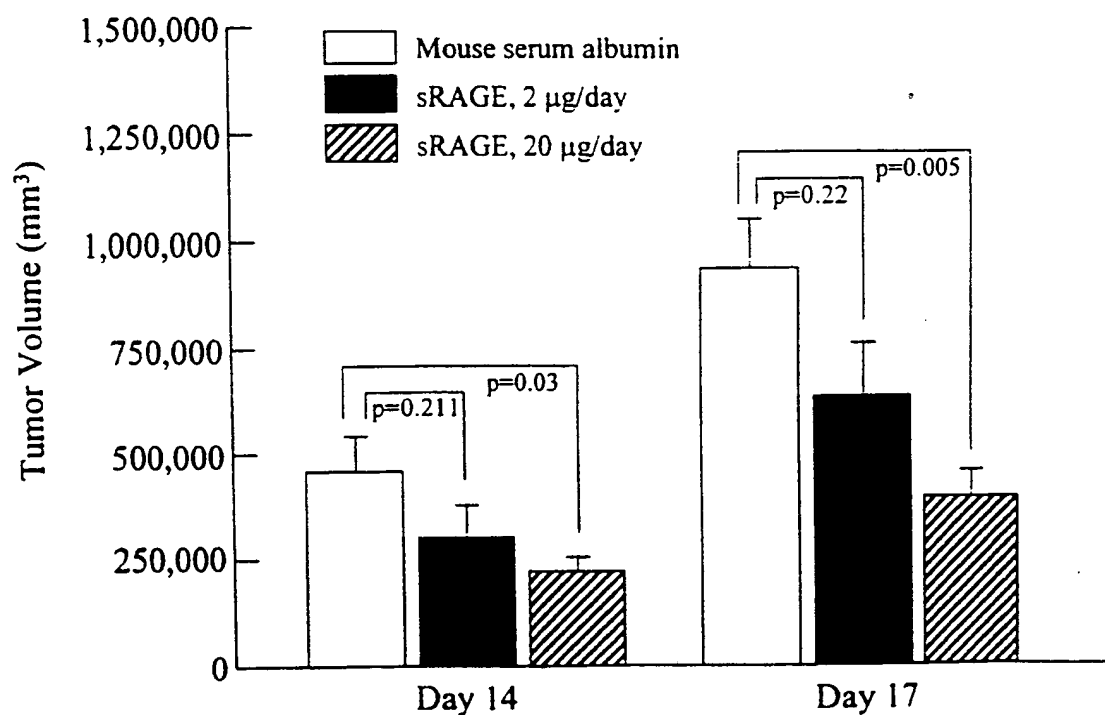


FIG. 4B



5/15

FIG. 5A

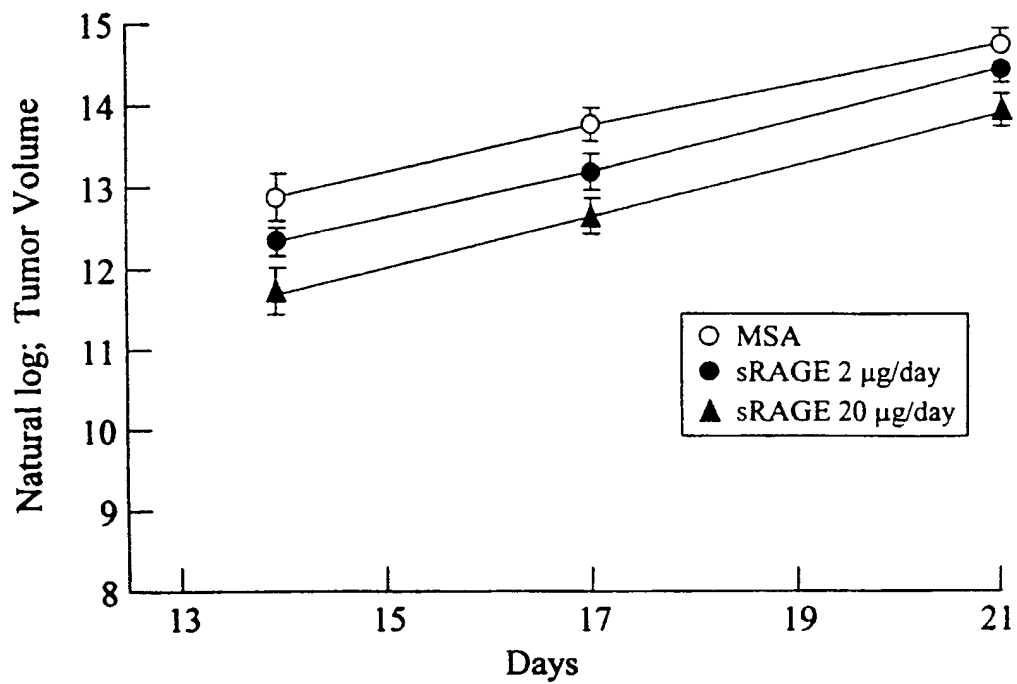
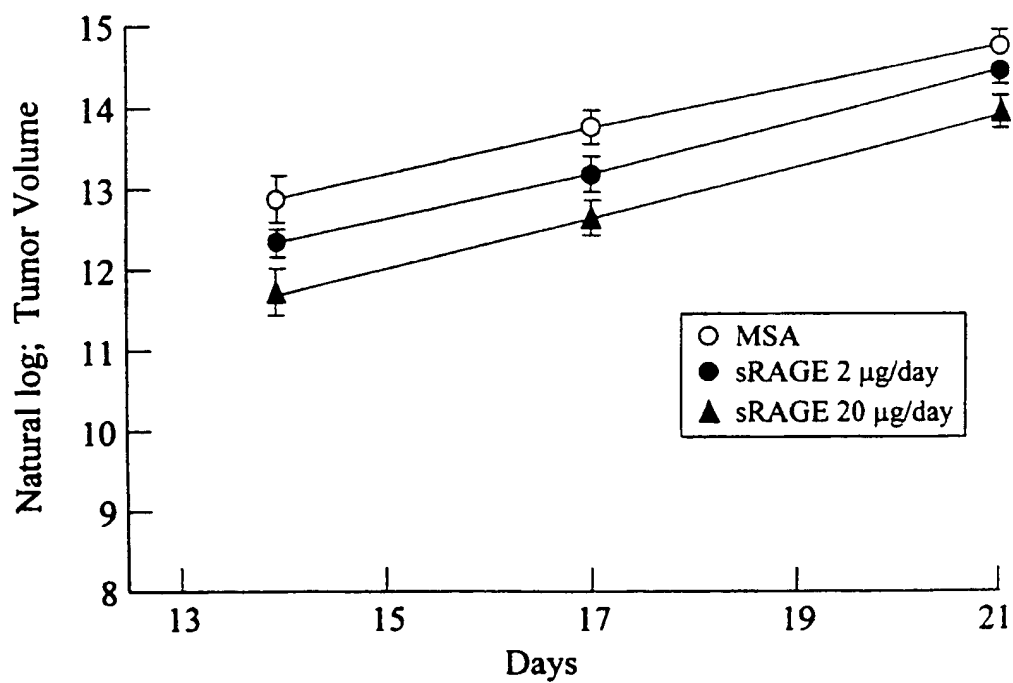


FIG. 5B



6/15

FIG. 6A

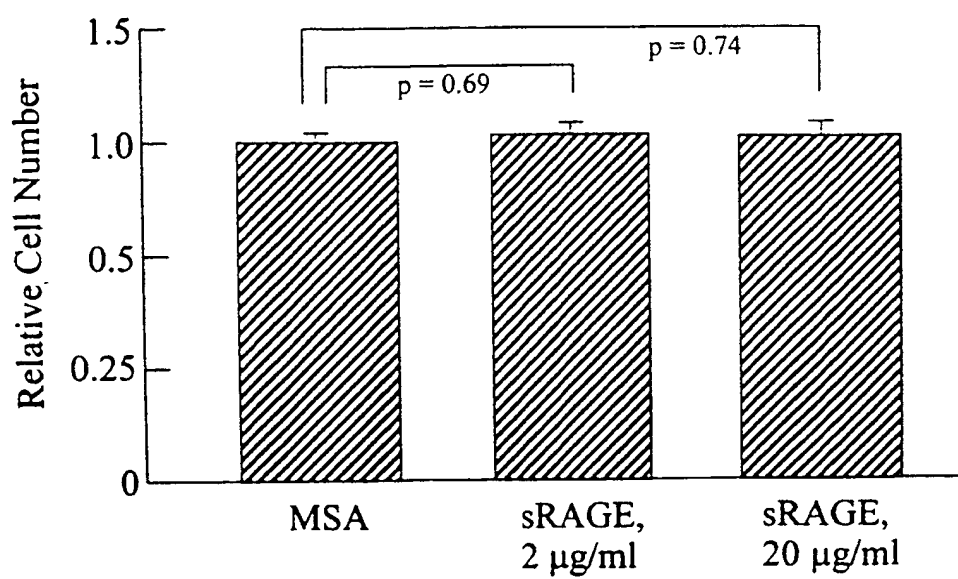
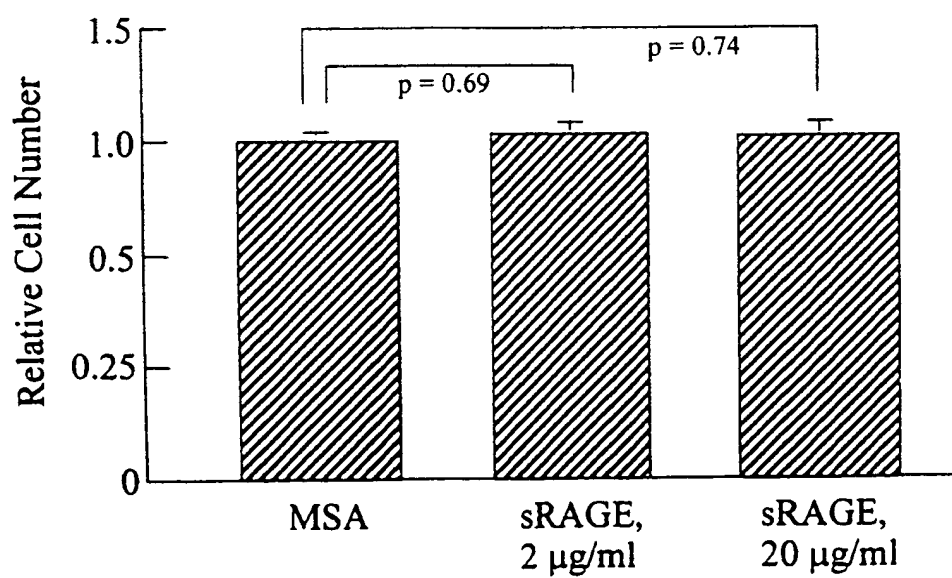


FIG. 6B



7/15

FIG. 7A-1

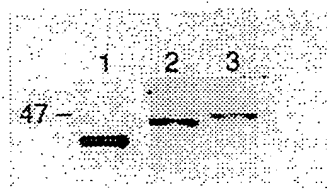
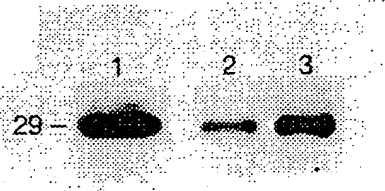
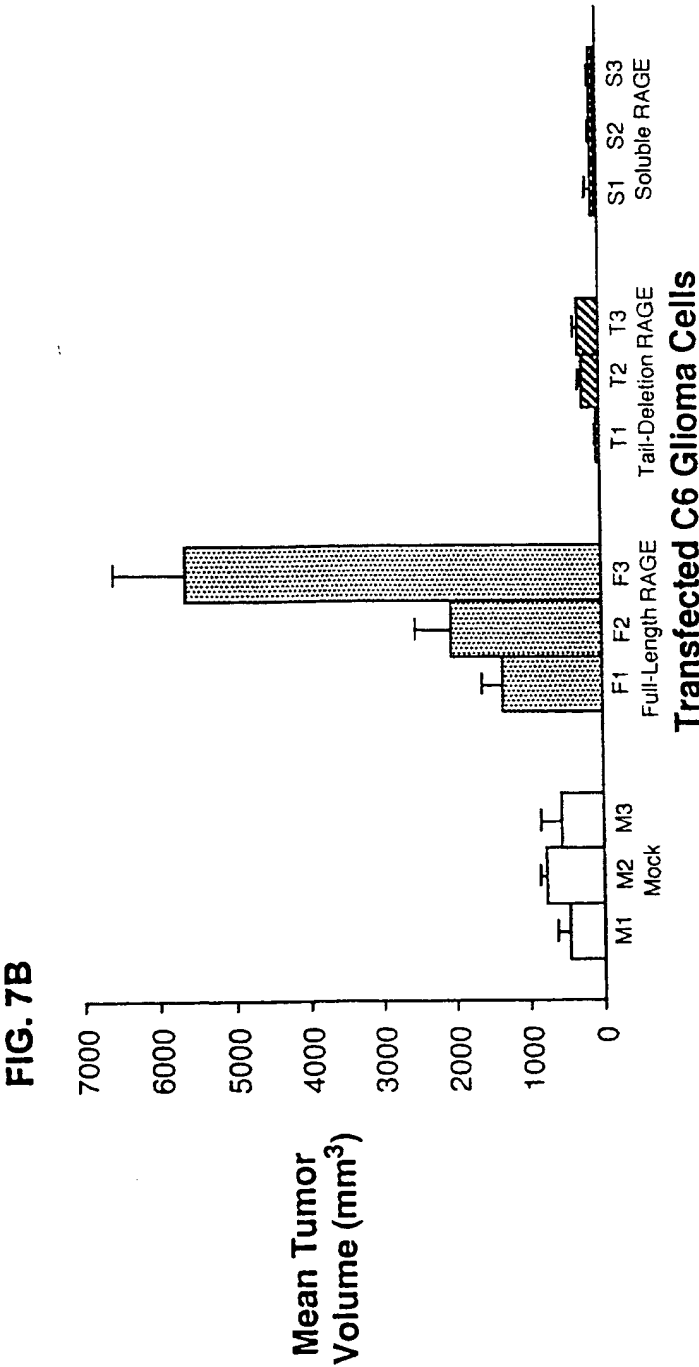


FIG. 7A-2





9/15

FIG. 7C



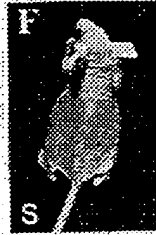
FIG. 7D



FIG. 7E



FIG. 7F



10/15

FIG. 7H

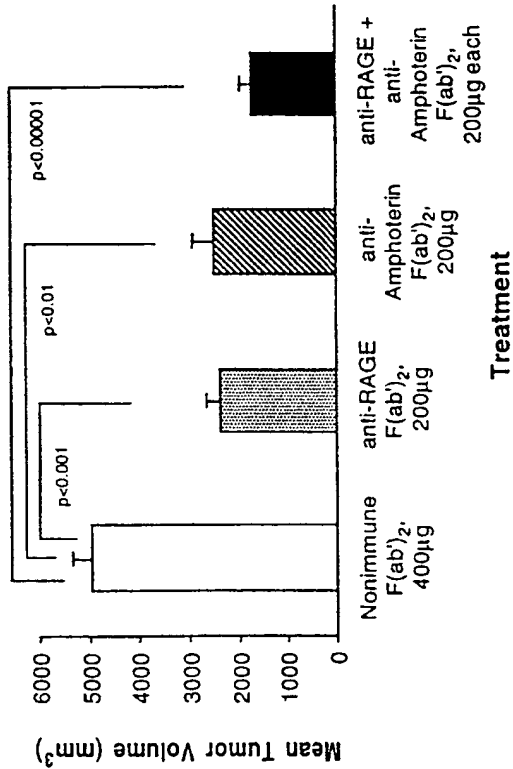
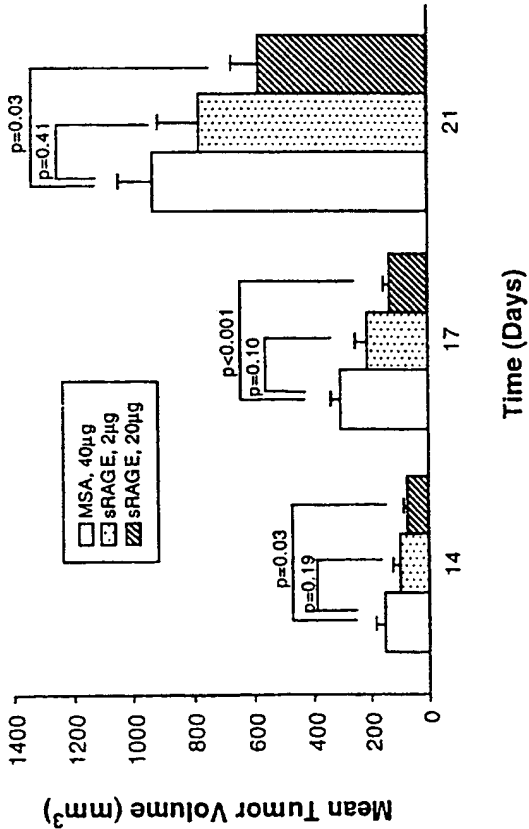
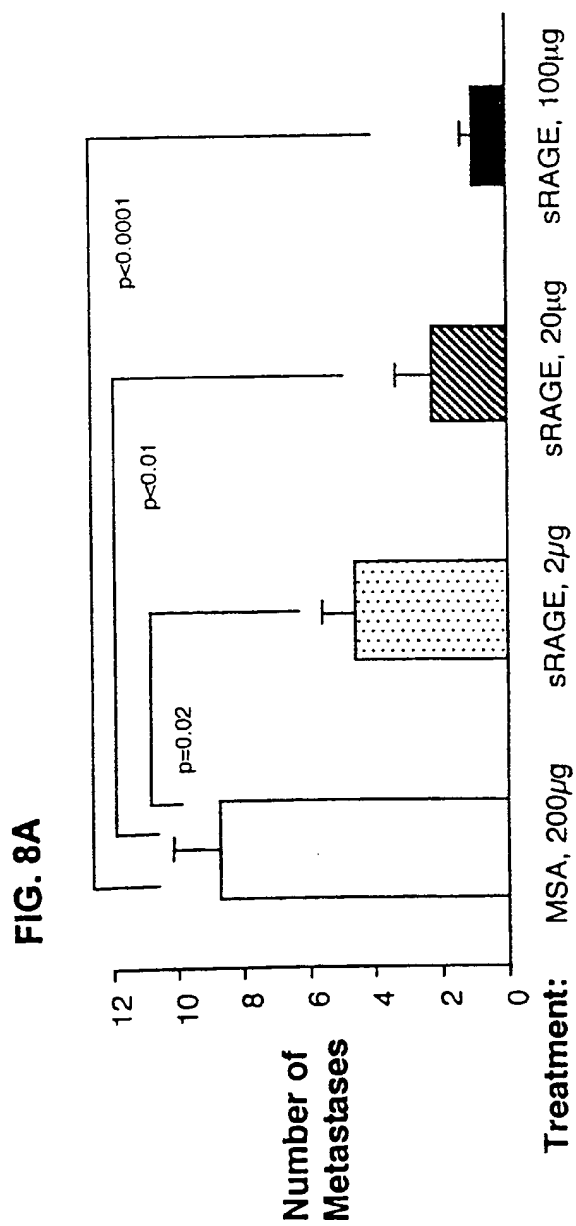


FIG. 7G



11/15



12/15

FIG. 8B FIG. 8C FIG. 8D FIG. 8E

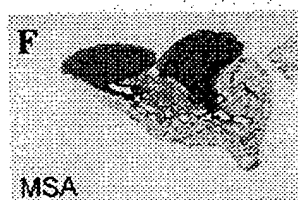
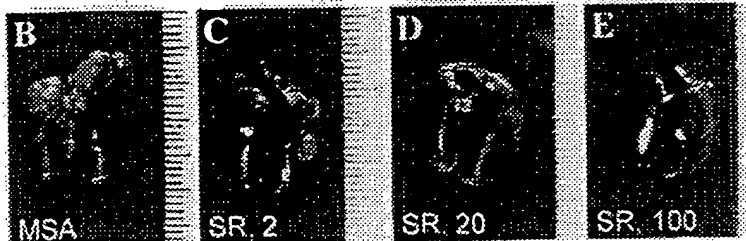


FIG. 8F



FIG. 8G

FIG. 9A

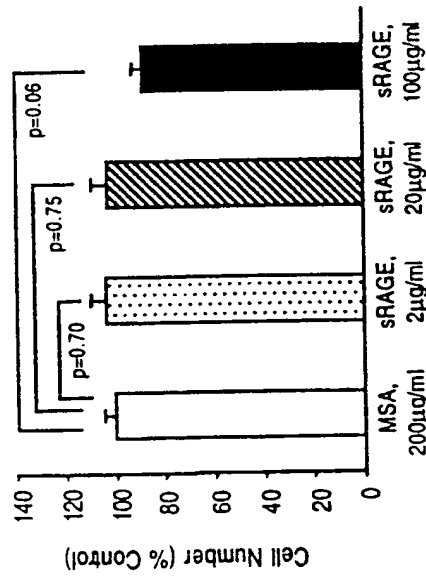


FIG. 9B

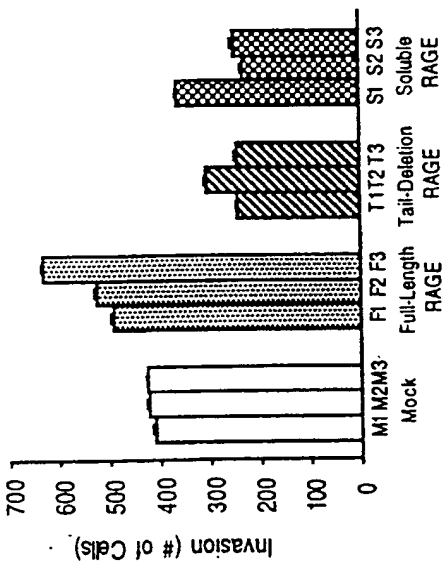


FIG. 9C

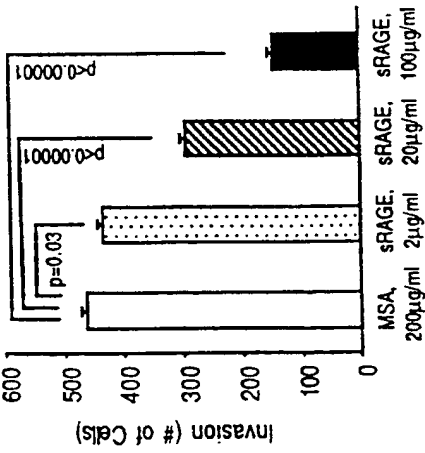


FIG. 9D

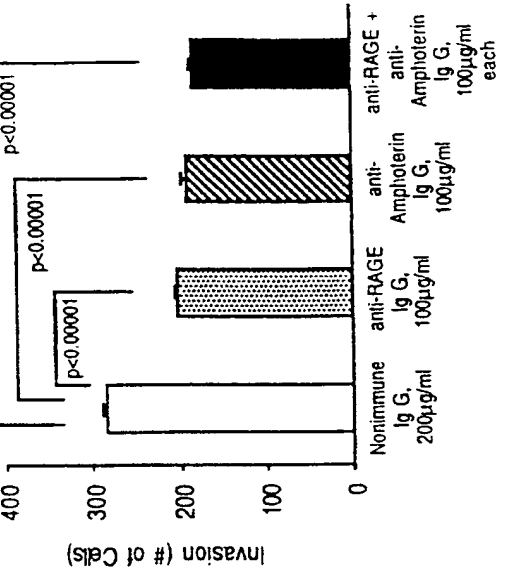


FIG. 9F

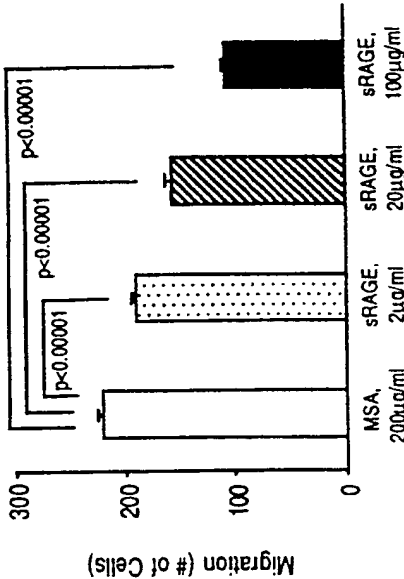


FIG. 9E

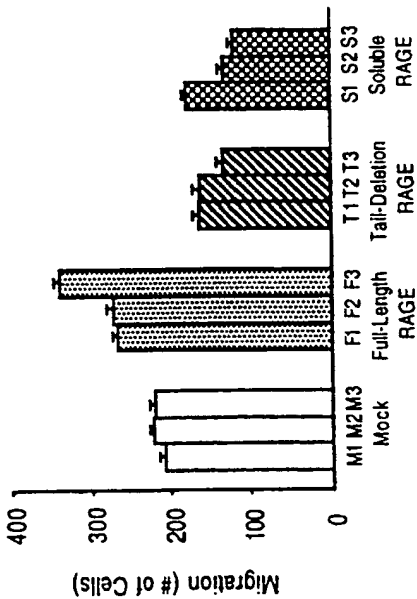
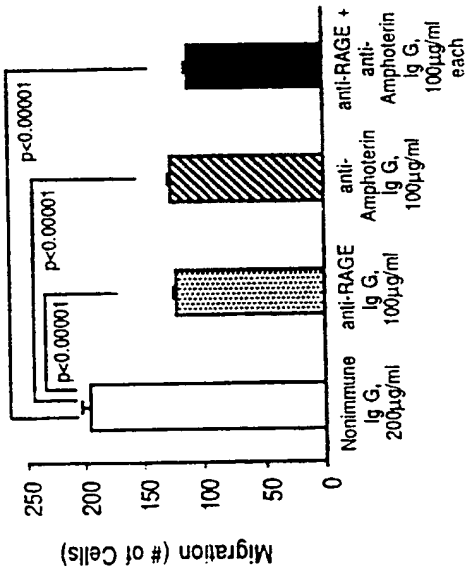
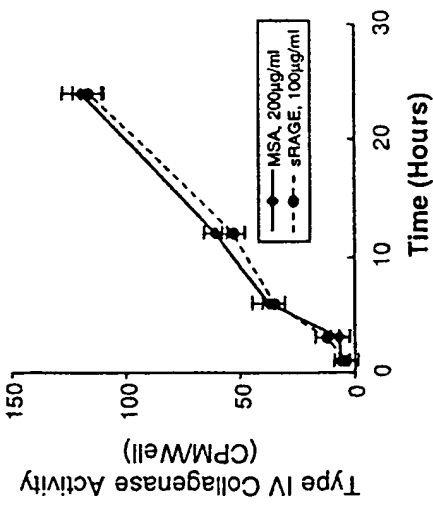
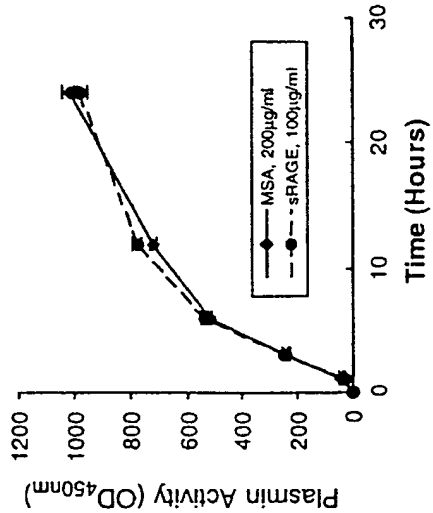
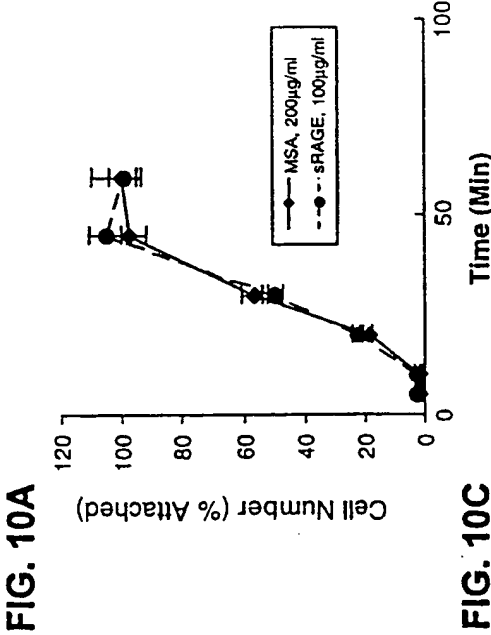
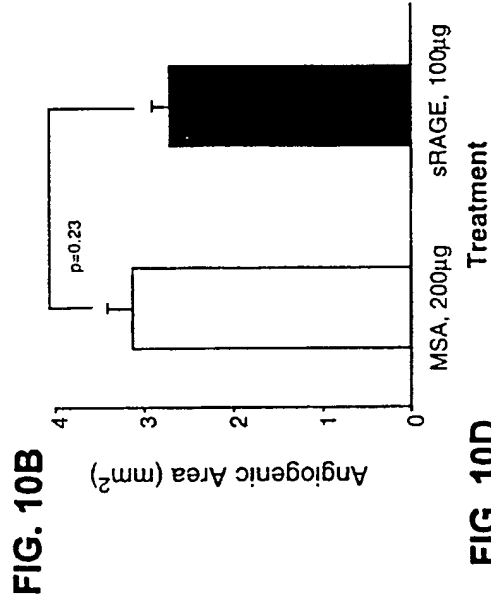


FIG. 9G





SEQUENCE LISTING

<110> The Trustees of Columbia University in the City of

<120> A Method for Inhibiting Tumor Invasion or Spreading in
a Subject

<130> 0575/55424-A-PCT

<140>

<141>

<150> 09/062,365

<151> 1998-04-17

<160> 10

<170> PatentIn Ver. 2.0 - beta

<210> 1

<211> 332

<212> PRT

<213> Homo sapiens

<400> 1

Ala Gln Asn Ile Thr Ala Arg Ile Gly Glu Pro Leu Val Leu Lys Cys
1 5 10 15

Lys Gly Ala Pro Lys Lys Pro Pro Gln Arg Leu Glu Trp Lys Leu Asn
20 25 30

Thr Gly Arg Thr Glu Ala Trp Lys Val Leu Ser Pro Gln Gly Gly Gly
35 40 45

Pro Trp Asp Ser Val Ala Arg Val Leu Pro Asn Gly Ser Leu Phe Leu
50 55 60

Pro Ala Val Gly Ile Gln Asp Glu Gly Ile Phe Arg Cys Gln Ala Met
65 70 75 80

Asn Arg Asn Gly Lys Glu Thr Lys Ser Asn Tyr Arg Val Arg Val Tyr
85 90 95

Gln Ile Pro Gly Lys Pro Glu Ile Val Asp Ser Ala Ser Glu Leu Thr
100 105 110

Ala Gly Val Pro Asn Lys Val Gly Thr Cys Val Ser Glu Gly Ser Tyr
115 120 125

Pro Ala Gly Thr Leu Ser Trp His Leu Asp Gly Lys Pro Leu Val Pro

130 135 140
 Asn Glu Lys Gly Val Ser Val Lys Glu Gln Thr Arg Arg His Pro Glu
 145 150 155 160
 Thr Gly Leu Phe Thr Leu Gln Ser Glu Leu Met Val Thr Pro Ala Arg
 165 170 175
 Gly Gly Asp Pro Arg Pro Thr Phe Ser Cys Ser Phe Ser Pro Gly Leu
 180 185 190
 Pro Arg His Arg Ala Leu Arg Thr Ala Pro Ile Gln Pro Arg Val Trp
 195 200 205
 Glu Pro Val Pro Leu Glu Glu Val Gln Leu Val Val Glu Pro Glu Gly
 210 215 220
 Gly Ala Val Ala Pro Gly Gly Thr Val Thr Leu Thr Cys Glu Val Pro
 225 230 235 240
 Ala Glu Pro Ser Pro Gln Ile His Trp Met Lys Asp Gly Val Pro Leu
 245 250 255
 Pro Leu Pro Pro Ser Pro Val Leu Ile Leu Pro Glu Ile Gly Pro Gln
 260 265 270
 Asp Gln Gly Thr Tyr Ser Cys Val Ala Thr His Ser Ser His Gly Pro
 275 280 285
 Gln Glu Ser Arg Ala Val Ser Ile Ser Ile Ile Glu Pro Gly Glu Glu
 290 295 300
 Gly Pro Thr Ala Gly Ser Val Gly Gly Ser Gly Leu Gly Thr Leu Ala
 305 310 315 320
 Leu Ala Leu Gly Ile Leu Gly Gly Leu Gly Thr Ala
 325 330

<210> 2

<211> 22

<212> PRT

<213> Homo sapiens

<400> 2

Met Ala Ala Gly Thr Ala Val Gly Ala Trp Val Leu Val Leu Ser Leu
 1 5 10 15

Trp Gly Ala Val Val Gly
 20

<210> 3
<211> 39
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide, peptide analog or non-natural peptide

<400> 3
Asp Ala Glu Phe Arg Asp Ser Gly Tyr Glu Val His His Gln Lys Leu
1 5 10 15
Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly
20 25 30
Leu Met Val Gly Gly Val Val
35

<210> 4
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide, peptide analog or non-natural peptide

<400> 4
Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu Met
1 5 10

<210> 5
<211> 30
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide, peptide analog or non-natural peptide

<400> 5
Ala Gln Asn Ile Thr Ala Arg Ile Gly Glu Pro Leu Val Leu Lys Cys
1 5 10 15
Lys Gly Ala Pro Lys Lys Pro Pro Gln Arg Leu Glu Trp Lys

20

25

30

<210> 6
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide, peptide analog or non-natural peptide

<400> 6
Ala Gln Asn Ile Thr Ala Arg Ile Gly Glu
1 5 10

<210> 7
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 7
acactgcagt cggagctaata 20

<210> 8
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 8
aagatgaccc caatgagcag 20

<210> 9
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 9
aagaagtgct cagagaggtg 20

<210> 10
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 10
taaggctgct tgtcatctgc

20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08427

| | | |
|--|--|--|
| A. CLASSIFICATION OF SUBJECT MATTER IPC(6) :C12N 15/63, 5/10, 15/09, 15/79, 5/00; A61K 48/00, 38/00 US CL :435/ 375, 325, 320.1, 7.2; 514/2, 44 According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/ 375, 325, 320.1, 7.2; 514/2, 44 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, STN, BIOSIS, MELINE, EMBASE, CANCERLIT search terms: rage, receptor glycation, endproducts | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| Y,P | WO 98/22138 A1 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 28 May 1998, see entire patent. | 1-26, 31-41 |
| Y | HORI et al. 'The Receptor for Advanced Glycation Endproducts: Implications for the development of diabetic vascular disease. Fundam. Clin. Cardiol.' In: The Endothelium in Clinical Practice. January 1997, Chapter 11, pages 311-329, see page 311. (Ed. Rubani and Dzau), Marcel Dekker, Inc. | 1-26, 31-41 |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex. | | |
| * "A" "B" "L" "O" "P" | Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier document published on or after the international filing date document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *G* document member of the same patent family |
| Date of the actual completion of the international search 29 JULY 1999 | | Date of mailing of the international search report 19 AUG 1999 |
| Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230 | | Authorized officer Karen M. Hauda JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX Telephone No. (703) 308-0196 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08427

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| Y | CHEN et al. Amyloid-beta peptide- receptor for Advanced Glycation Endproduct Interaction Elicits Neuronal Expression of Macrophage-Colony Stimulating Factor: a proinflammatory pathway in Alzheimer Disease. Proc. Natl. Acad. Sci. 13 May 1997, Vol. 94, No. 10, pages 5296-5301, see entire article. | 1-26, 31-41 |
| Y | SCHMIDT et al. Receptor-dependent Hyperfibrinogenemia in Diabetic Mice: Reversal by blockade of the receptor for advanced glycation endproducts. Circulation. 15 October 1995, Vol. 92, No. 8, page 1694, see abstract 3333. | 1-26, 31-41 |
| Y, P | SCHMIDT et al. The V-domain of Receptor for Advanced Glycation Endproducts (RAGE) Mediates Binding of AGEs: A novel target for therapy of diabetic complications. Circulation. 21 October 1997, Vol. 96, No. 8 Suppl., page 137, see abstract 194. | 1-26, 31-41 |
| Y | HORI et al. The Receptor for Advanced Glycation Endproducts (RAGE) is a Cell Surface Receptor for Amphoterin in the Developing Central Nervous System (CNS) to Promote Neurite Outgrowth. FASEB J. 1995, Vol. 9, No. 3, page A382, see abstract 2212. | 1-26, 31-41 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08427

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 27-30
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 27-30 contain nucleotide sequences which can not be searched other than by sequence search. No CRF in this case has been filed.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.